Biochimica et Biophysica Acta, 511 (1978) 10—22

BBA 78076

THE EFFECT OF SODIUM ON INORGANIC PHOSPHATE- AND *p*-NITROPHENYL PHOSPHATE-FACILITATED OUABAIN BINDING TO ($Na^+ + K^+$)-ACTIVATED ATPase

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(Received December 28th, 1977)

Summary

The effect of the hydrolysis product P_i and the artificial substrate *p*-nitrophenyl phosphate (*p*-nitrophenyl-*P*) on ouabain binding to ($Na^+ + K^+$)-activated ATPase was investigated.

The hypothesis that (Mg^{2+} + *p*-nitrophenyl-*P*)-supported ouabain binding might be due to P_i release and thus ($Mg^{2+} + P_i$)-supported could not be confirmed.

The enzyme · ouabain complexes obtained with different substrates were characterized according to their dissociation rates after removal of the ligands facilitating binding. The character of the enzyme · ouabain complex is determined primarily by the monovalent ion present during ouabain binding, but, qualitatively at least, it is immaterial whether binding was obtained with *p*-nitrophenyl phosphate or P_i.

The presence or absence of Na^+ during binding has a special influence upon the character of the enzyme · ouabain complex. Without Na^+ and in the presence of Tris ions the complex obtained with ($Mg^{2+} + P_i$) and that obtained with (Mg^{2+} + *p*-nitrophenyl-*P*) behaved in a nearly identical manner, both exhibiting a slow decay. High Na^+ concentration diminished the level of P_i-supported ouabain binding, having almost no effect on *p*-nitrophenyl phosphate-supported binding. Both enzyme · ouabain complexes, however, now resembled the form obtained with (Na^+ + ATP), as judged from their dissociation rates and the K^+ sensitivity of their decay. The complexes obtained at a high Na^+ concentration underwent a very fast decay which could be slowed considerably after adding a low concentration of K^+ to the resuspension medium. The most stable enzyme · ouabain complex was obtained in the presence of Tris ions only, irrespective of whether *p*-nitrophenyl phosphate or P_i facilitated complex formation. The presence of K^+ gave rise to a complex whose dissociation rate was intermediate between those of the complexes obtained in the presence of Tris and a high Na^+ concentration.

It is proposed that the different ouabain dissociation rates reflect different

reactive states of the enzyme. The resemblance between the observations obtained in phosphorylation and ouabain binding experiments is pointed out.

Introduction

Cardiac glycosides are specific inhibitors of the ($Na^+ + K^+$)-activated ATPase, but binding of, e.g., ouabain takes place only under special conditions. At a given low ouabain concentration a high degree of binding is obtained with ($Mg^{2+} + Na^+ + ATP$) or with ($Mg^{2+} + P_i$) [1—4]. The substrate ATP and its hydrolysis product P_i are known to phosphorylate the ($Na^+ + K^+$)-ATPase under the specific conditions, and it has been suggested that phosphorylation is a prerequisite for ouabain binding [1,2]. Merely binding of ATP or non-hydrolysable substrates excludes ouabain binding [5—7].

The ($Na^+ + K^+$)-ATPase also possesses a neutral K^+-activated phosphatase activity (e.g. *p*-nitrophenylphosphatase activity) which has been proposed to reflect a terminal K^+-dependent hydrolytic step of the overall ATPase reaction [8]. Phosphorylation of ($Na^+ + K^+$)-ATPase by *p*-nitrophenyl phosphate in the presence of ouabain has been demonstrated by Inturrisi and Titus [9], and finally it has been demonstrated that phosphatase substrates also support ouabain binding and best so in the presence of Na^+ [10,11].

Whether the final enzyme · ouabain complex is always phosphorylated or not is an unanswered question, but the common denominator in the three situations with ATP-, P_i- and *p*-nitrophenyl phosphate-supported ouabain binding is that phosphorylation of the enzyme takes place. Even assuming that phosphorylation is a prerequisite for ouabain binding, the enzyme · ouabain complex need not be different in the three situations, if the character of the complex was determined by the bound ouabain. However, that the reactive state of the enzyme · ouabain complex obtained with ($Mg^{2+} + P_i$) and ($Mg^{2+} + Na^+ + ATP$) differ is indicated by the very different dissociation rate of the two complexes as measured after removal of the ligands which has led to the binding of ouabain [11,12]. An intermediate rate of dissociation is said to characterize the (Mg^{2+} + *p*-nitrophenyl-*P*)-supported complex [13].

The present study of the different kinetic behaviour was based upon the following two assumptions: (1) Ouabain binding as supported by *p*-nitrophenyl phosphate might be due to P_i released from hydrolysis of *p*-nitrophenyl phosphate, or alternatively, the *p*-nitrophenyl phosphate-supported complex might not be very different from the P_i-supported one because of the low free energy of hydrolysis of *p*-nitrophenyl phosphate. (2) The presence of Na^+ in one of the facilitating mixtures (with ATP) might be important for the character of the enzyme · ouabain complex since it has been demonstrated in experiments with *N*-ethylmaleimide and trypsin that the reactive state of the ($Na^+ + K^+$)-activated ATPase is affected by monovalent cations [14,15].

A preliminary report on the present results has been given elsewhere [16].

Materials and Methods

Enzyme preparation of ($Na^+ + K^+$)-ATPase from ox brain was prepared as described by Klodos et al. [17]. The specific activity was 167—202 μmol

P_i/mg protein per h and the preparations were practically deprived of ATPase activity not dependent on $Na^+ + K^+$.

Uniformly labelled [^{3}H]ouabain (G-strophanthin) was obtained from New England Nuclear Corp. and was purified as described elsewhere [7]. The purified [^{3}H]ouabain was diluted with unlabelled ouabain (Merck) to specific activities in the range 25—200 Ci/mol.

The chemicals used were of analytical grade. The disodium salt of *p*-nitrophenyl phosphate (Merck) was converted to the Tris salt by chromatography on Dowex 50 W in the acidic form and neutralization of the effluent with Tris base. P_i solutions were prepared from orthophosphoric acid (Merck, Darmstadt) neutralized with 2-amino-2-methyl-1,3-propandiol (Fluka). Inosin (Merck, Darmstadt), nucleoside phosphorylase and xanthine oxydase (in 3.2 M $(NH_4)_2SO_4$, Boehringer Mannheim) were used to trap inorganic phosphate released from *p*-nitrophenyl phosphate. In typical experiments in which the question of *p*-nitrophenyl phosphate or P_i facilitation of ouabain binding was examined the time course of binding was studied with 0.1—0.2 mg ATPase protein, 5 mM Mg^{2+}, 1 mM *p*-nitrophenyl phosphate, $2.5 \cdot 10^{-8}$ M [^{3}H]ouabain, 25 mM inosin, 0.44 μg nucleoside phosphorylase/ml (8.8 munits/ml) and 11 μg xanthine oxidase/ml incubation mixture (4.4 munits/ml). P_i was assayed by the method described by Ottolenghi [18].

For [^{3}H]ouabain binding the reagents were mixed at 0°C and the binding was then achieved by incubation at 37°C. Bound and non-bound ouabain was separated by filtration through Sartorius or Seitz membrane filters (pore size 0.6 μm) with suction. Aliquots of filtrates obtained immediately after mixture of the reagents at 0°C were taken for determination of total ^{3}H radioactivity. To determine non-bound radioactivity during the incubation at 37°C aliquots of filtrates taken at this step were used. In experiments where the dissociation of the enzyme · ouabain complex was studied (the binding being initially 100%) the total ^{3}H radioactivity of the dissociation medium was determined from unfiltered samples. The difference between ^{3}H radioactivity of the first and the second filtrate or between an unfiltered sample and the filtrate was taken to be bound [^{3}H]ouabain. A Triton X-114/xylene scintillation mixture and a Packard liquid spectrometer were used for counting. The results were corrected for quenching by external standardization.

For characterization of enzyme · ouabain complexes obtained with different incubation media, the ouabain exchange was blocked by cooling to 0°C. The enzyme was spun down and washed twice in a Beckman L2-65B ultracentrifuge at 100 000 × *g* in the cold. The release of previously bound ouabain was finally measured by the filtration technique after resuspension at 0°C of the pellet in one of the media indicated in the legends to the figures and then warming the mixture to 37°C. A high concentration (10^{-3} M) of unlabelled ouabain was always present in the resuspension medium to minimize rebinding of [^{3}H]ouabain.

[^{3}H]Ouabain released during the resuspension in the cold was a minute fraction (<3%) of the total bound ouabain. This minute fraction was subtracted, and the remaining activity was used as the 100% initial binding value in the figures. The dissociation curves shown represent single experiments, but they have been reproduced several times.

Results

Ouabain binding with Mg^{2+} and p-nitrophenyl phosphate

The rate of ouabain binding in the presence of (Mg^{2+} + P_i) or (Mg^{2+} + Na^+ + ATP) is remarkably slow [3,4]. In a preliminary study, the time course of ouabain binding in the presence of (Mg^{2+} + *p*-nitrophenyl-*P*) was examined. In Fig. 1 it is seen that the rate of ouabain binding is still slower with (Mg^{2+} + *p*-nitrophenyl-*P*) than with (Mg^{2+} + P_i). Even without the addition of K^+, the enzyme preparation has some phosphatase activity and will thus produce some P_i. Hence, the very slow rate of ouabain binding in the presence of *p*-nitrophenyl phosphate raises the question as to whether the rate-limiting factor for the binding process might be the release of P_i from *p*-nitrophenyl phosphate rather than a more direct involvement of *p*-nitrophenyl phosphate itself. If the enzyme is incubated from the beginning with the P_i and *p*-nitrophenyl phosphate concentrations found after 3 h of incubation with 1 mM *p*-nitrophenyl phosphate, an acceleration of the rate of binding is achieved as seen from Fig. 1. The equilibrium binding level is also raised somewhat, but is still lower than that obtained with only 1 mM P_i. The time course and the equilibrium binding level were not affected by *p*-nitrophenol. By addition of *p*-nitrophenyl phosphate at equilibrium of ouabain binding obtained with P_i, a release of previously bound ouabain is seen (Fig. 1).

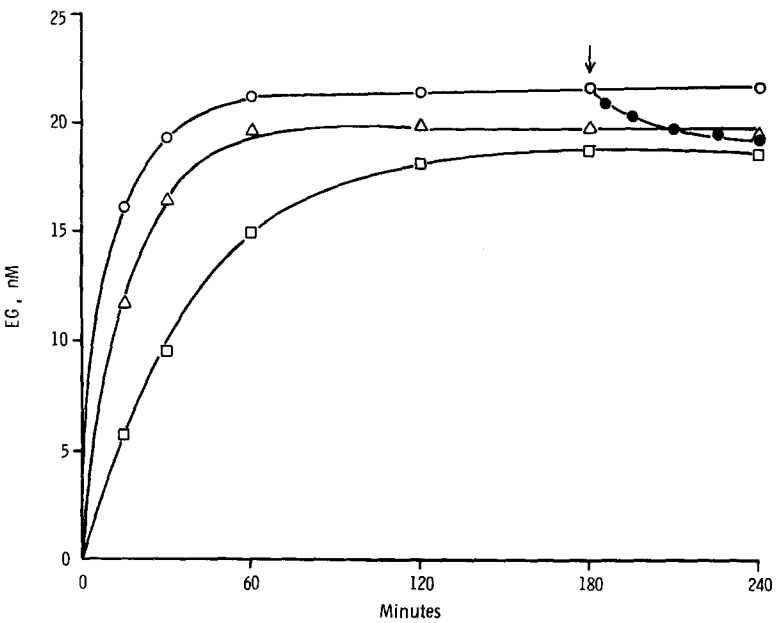

Fig. 1. Time course of ouabain binding with (Mg^{2+} + P_i) or (Mg^{2+} + *p*-nitrophenyl-*P*). Enzyme (0.21 mg protein/ml, ouabain-sensitive activity 167 μmol P_i/mg protein per h) was incubated with 5 mM Mg^{2+}, 35 mM Tris (pH 7.25), $2.5 \cdot 10^{-8}$ M [^{3}H]ouabain and ○———○, 0.3 mM P_i; □———□, 1 mM *p*-nitrophenyl phosphate or △———△, 0.3 mM P_i + 0.7 mM *p*-nitrophenyl phosphate. In a parallel experiment *p*-nitrophenyl phosphate was added after 180 min (↓) to the P_i incubation medium to give a final concentration of 1 mM (●———●). Ouabain binding (EG) was determined as described in Materials and Methods.

These preliminary experiments might indicate that ouabain binding is supported by P_i formed from *p*-nitrophenyl phosphate, whereas *p*-nitrophenyl phosphate as such is inhibitory to ouabain binding. To examine more directly whether released P_i is indeed responsible for the ouabain binding obtained with *p*-nitrophenyl phosphate, another approach was attempted. By adding nucleoside phosphorylase and inosin to the (Mg^{2+} + *p*-nitrophenyl-*P*) binding system, inosin + released P_i should be converted to ribose phosphate and hypoxanthine. Removal of hypoxanthine by xanthine oxidase shifts the equilibrium of the process in favour of the cleavage of inosin rather than its synthesis. Control experiments were without inosin added. In such experiments the concentration of free P_i was decreased by more than 50%. The rate and final level of ouabain binding was somewhat depressed as was the binding when only Mg^{2+} was used for binding promotion. However, since incubation with the complete P_i-trapping system led to a nearly parallel decrease in ($Na^+ + K^+$)-activated ATPase activity, it is dubious whether P_i is the facilitating ligand for the (Mg^{2+} + *p*-nitrophenyl-*P*)-supported ouabain binding. The much higher binding with *p*-nitrophenyl phosphate than with P_i in the presence of Na^+ (see next section) seems to support this view.

Characterization of the enzyme · ouabain complexes formed with (Mg^{2+} + P_i) and (Mg^{2+} + p-nitrophenyl-P) from their dissociation rates

The idea that different enzyme · ouabain complexes are obtained with different facilitating ligands for ouabain binding is based on the observations of Akera and Brody [12] and of Allen et al. [13]. Since the binding process is extremely slow and practically irreversible at 0°C, the facilitating ligands can be removed by centrifugation at this temperature after ouabain binding at 37°C. Upon resuspension at 37°C in buffer alone the dissociation rate of ouabain from the (Mg^{2+} + P_i)-supported complex is very much slower than that of the (Mg^{2+} + Na^+ + ATP)-supported complex. By addition of K^+ to the resuspension medium the latter complex is stabilized to give the same slow dissociation rate as the former, which is not affected by K^+. The two enzyme · ouabain complexes are now equal as judged from their rates of decay.

Preliminary experiments in which the dissociation rate of ouabain from the (Mg^{2+} + *p*-nitrophenyl-*P*)-supported complex was compared to that of the (Mg^{2+} + P_i)-supported complex, could not confirm the observations of others [11] that the rate of decay was faster with a complex formed with *p*-nitrophenyl phosphate than with P_i. The ouabain binding levels were 130 and 195 pmol per mg protein after 1 h incubation with $5 \cdot 10^{-8}$ M [^{3}H]ouabain, and either 5 mM Mg^{2+} + 1 mM *p*-nitrophenyl phosphate (Tris salt) or 5 mM Mg^{2+} + 0.3 mM P_i, respectively. In spite of the different binding levels achieved, the time course of the two off-processes after washing with and resuspension of the enzyme · ouabain complexes in dilute Tris buffer was quite identical and slow (Fig. 2). Also, no effect of addition of K^+ to the resuspension media was observed with either of the complexes.

The main difference between the present study and that referred to above [11] was the use of Tris salt in the present study and of sodium salt of *p*-nitrophenyl phosphate in theirs. This led to a study of the effect of alkali metal ions present during the binding period on the character of the enzyme · ouabain

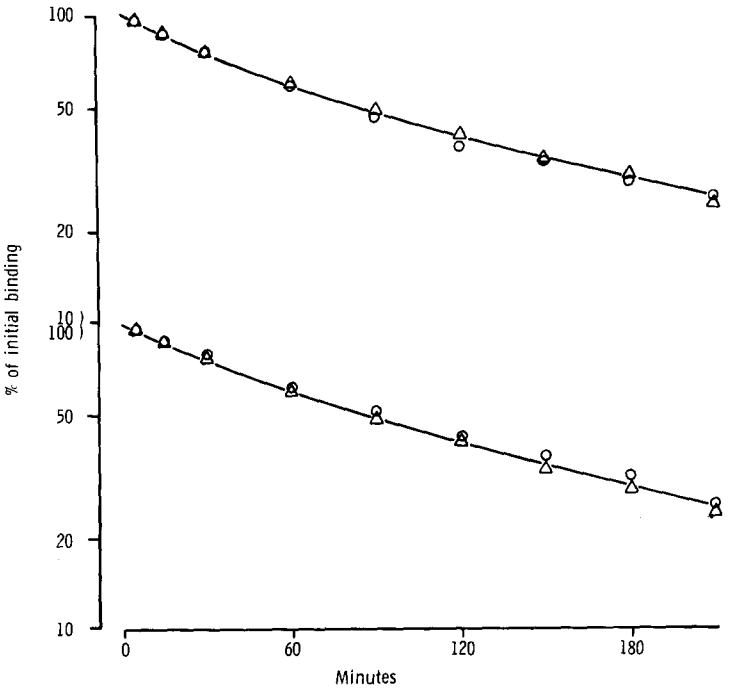

Fig. 2. Comparison of ouabain dissociation rates after (Mg^{2+} + P_i)- and (Mg^{2+} + *p*-nitrophenyl-*P*)-facilitated ouabain binding. Binding was achieved during 1 h incubation of 0.21 mg protein/ml with 5 mM Mg^{2+}, 1 mM *p*-nitrophenyl phosphate, 40 mM Tris and $5 \cdot 10^{-8}$ M [^{3}H]ouabain (upper curve) or 5 mM Mg^{2+}, 0.3 mM P_i, 40 mM Tris and $5 \cdot 10^{-8}$ M [^{3}H]ouabain (lower curve). Incubation was terminated and the enzyme · ouabain complex isolated at 0°C as described in Materials and Methods. After resuspension in 4 mM Tris (△) or 4 mM Tris + 2 mM K^+ (○) release of [^{3}H]ouabain at 37°C was determined as described and expressed as percent of initial binding after wash and resuspension at 0°C. 10^{-3} M ouabain was present during the dissociation.

complex formed. It was found that the type of enzyme · ouabain complex obtained was heavily influenced by the presence of Na^+, K^+ or Tris, but, was independent of whether binding of ouabain was induced by P_i or *p*-nitrophenyl phosphate. The amount of ouabain bound, however, is very different with P_i and *p*-nitrophenyl phosphate in the presence of cations.

In (Mg^{2+} + P_i)-facilitated ouabain binding addition of K^+ to the medium decreased the equilibrium binding level, but even high concentrations of K^+ did not abolish binding. High enough concentrations of Na^+ seemed to exclude ouabain binding [4]. With $2 \cdot 10^{-7}$ M [^{3}H]ouabain plus 100 mM K^+, 100 mM Na^+ or 130 mM Tris (identical ionic strength), 95, 32 and 187 pmol of ouabain were bound per mg protein after 1 h of incubation in the three situations. The rate of decay of the enzyme · ouabain complexes tested under identical conditions with just dilute buffer appeared slow after binding with Tris, faster with K^+ and fastest with Na^+ (Fig. 3). None of the curves can be described as being single exponentials, especially not those which arose from Tris and Na^+. The Na^+ curve clearly contains a very fast component. Most remarkable, however, was the observation that the enzyme · ouabain complex obtained with (Mg^{2+} + P_i) plus 100 mM Na^+ now disclosed K^+ sensitivity during the dissociation. As

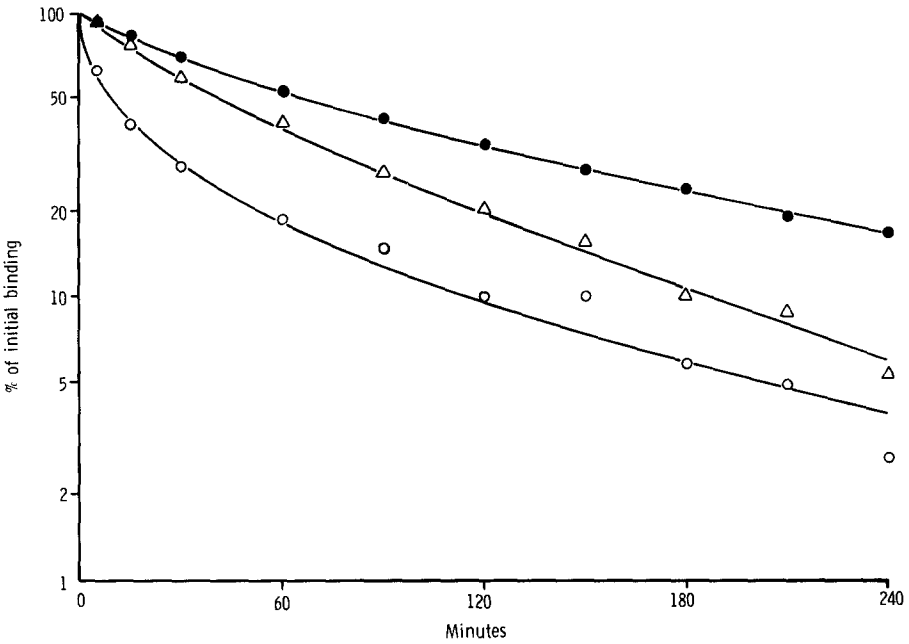

Fig. 3. Ouabain dissociation rates after (Mg^{2+} + P_i)-facilitated ouabain binding with Tris, Na^+ or K^+ present during binding. 0.21 mg protein/ml was incubated for 1 h with 3 mM Mg^{2+}, 3 mM P_i, 5 mM Tris (pH 7.25), $2 \cdot 10^{-7}$ M [^{3}H]ouabain, and o———o, 100 mM Na^+; △———△, 100 mM K^+ or •———•, 125 mM Tris. Ouabain binding was terminated and the enzyme · ouabain complex isolated at 0°C as described in Materials and Methods. After resuspension in 4 mM Tris + 10^{-3} M ouabain the release of [^{3}H]ouabain at 37°C was determined as described and expressed as percent of initial binding after wash and resuspension at 0°C.

seen from Fig. 4, the decay was much faster in Tris buffer than in Tris buffer + 2 mM K^+. With lower concentrations of Na^+ during the binding period the rate of decay was slower, whereas a further acceleration of the off-process was seen at higher Na^+ concentrations. However, in the latter situation the level of ouabain binding became low and made it difficult to determine the time course of complex dissociation with accuracy.

Independent of the situation during binding and release the final part of the dissociation curves are apparently parallel. By assuming that the curves obtained after binding in the presence of 100 mM Na^+ are composed of two independent and monoexponential parts and by subtracting the slower one from the compound curve a steep, apparently linear component is obtained. The rate of decay of the fast component is comparable to but still slower than the rate of dissociation of the enzyme · ouabain complex obtained with (Mg^{2+} + ATP) and 100 mM Na^+ (not shown).

It is dubious whether any of the curves of Figs. 2—4 are single exponentials. They may all contain a fast and a slow component. The equilibrium concentration of enzyme · ouabain complex obtained with (Mg + P_i) was much higher when Tris and K^+ was present during binding than when Na^+ was present (Table I). You could thus imagine that complex formation corresponding to the fast component is just selected in the presence of Na^+. This explanation

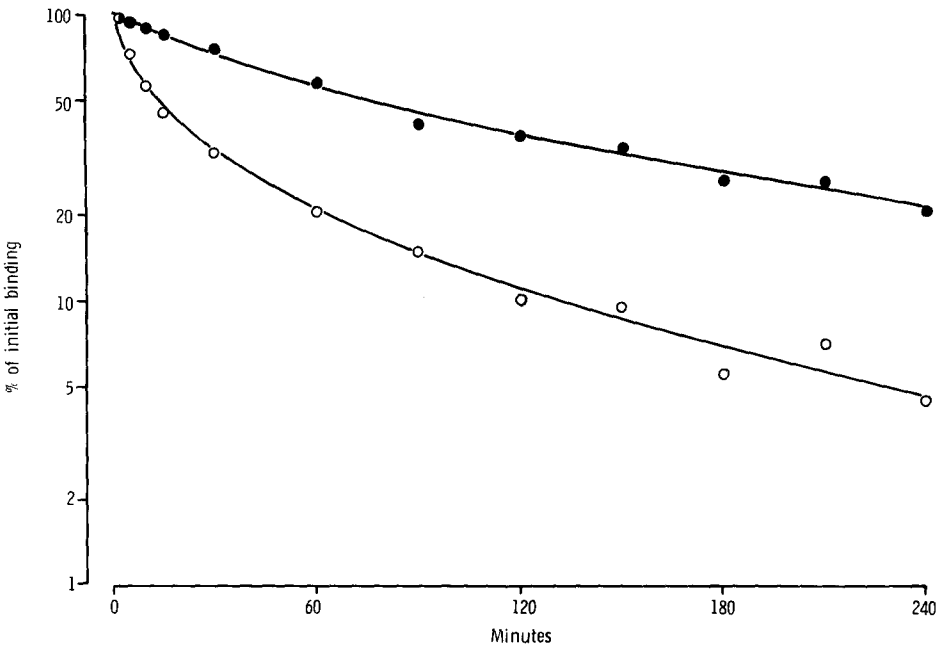

Fig. 4. Effect of K^+ on the dissociation rate after (Mg^{2+} + P_i)-facilitated ouabain binding with 100 mM Na^+ present during the binding. 0.21 mg protein/ml was incubated for 1 h with 3 mM Mg^{2+}, 3 mM P_i, 5 mM Tris (pH 7.25), 100 mM Na^+ and $2 \cdot 10^{-7}$ M [^{3}H]ouabain. Incubation was terminated and the enzyme · ouabain complex isolated at 0°C as described in Materials and Methods. 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 and expressed as percent of initial binding after wash and resuspension at 0°C.

does not hold, however, when experiments with *p*-nitrophenyl phosphate is included.

Na^+ inhibits the binding level of enzyme · ouabain complex much less when binding is obtained with (Mg^{2+} + *p*-nitrophenyl-*P*) than when it is obtained with (Mg^{2+} + P_i). In an experiment with 100 mM Na^+, 335 and 58 pmol of ouabain

TABLE I

SUMMARY OF [^{3}H]OUABAIN BINDING DATA REFERRED TO IN THE TEXT

Two different enzyme preparations with binding capacities of 365 and 502 mol per mg protein were used in (Mg^{2+} + P_i)- and (Mg^{2+} + *p*-nitrophenyl-*P*)-supported binding, respectively. An exception is the figure in brackets which represents ouabain binding to the enzyme with the higher capacity. Ouabain binding was measured after 1 h incubation with $2 \cdot 10^{-7}$ M [^{3}H]ouabain as described and binding capacity was determined from Scatchard plots of binding data [4,7].

mM cation present during binding (+ Tris, 5 mM)	pmol ouabain bound per mg protein in the	
	(Mg^{2+} + P_i)-supported pathway	(Mg^{2+} + *p*-nitrophenyl-*P*)-supported pathway
—	—	367
Tris, 125	187	47
Na^+, 100	32 (58)	335
K^+, 100	95	105

were bound per mg protein after incubation with $2 \cdot 10^{-7}$ M ouabain plus (Mg^{2+} + *p*-nitrophenyl-*P*) or (Mg^{2+} + P_i), respectively. A summary of the ouabain binding data obtained in different situations is shown in Table I. At a lower ouabain concentration it is seen from Fig. 5 that the ouabain equilibrium binding level obtained with (Mg^{2+} + *p*-nitrophenyl-*P*) decreased somewhat with increasing Na^+ concentration. However, if the ionic strength was kept constant by substituting Tris with Na^+, an increase in ouabain binding was obtained with increasing Na^+ concentration. The ion with the most spectacular inhibotory effect on the ouabain binding level is thus the Tris ion, and the facilitating effect of Na^+ is rather due to Tris-Na^+ competition. The reason for the much stronger inhibitory effect of Tris on (Mg^{2+} + *p*-nitrophenyl-*P*)-supported than on (Mg^{2+} + P_i)-supported ouabain binding [4] is unknown. This phenomenon, however, explains for the lower equilibrium binding level obtained with *p*-nitrophenyl phosphate than with P_i in the presence of 40 mM Tris in Fig. 1.

Although Na^+ seems to be rather indifferent as far as the ouabain binding level goes in (Mg^{2+} + *p*-nitrophenyl-*P*)-facilitated binding, the character of the complex obtained is very much changed at increasing Na^+ concentration during binding. In Fig. 6 it is seen that the dissociation rate tested in Tris buffer of the enzyme · ouabain complex obtained in the presence of 100 mM Na^+ is much faster than when Na^+ was omitted during binding. The rate of decay of the *p*-nitrophenyl phosphate-supported complex obtained with Na^+ is even faster than that of the P_i-supported complex formed in the presence of Na^+ (compare with Fig. 3) and a smaller fraction has a slow release of ouabain. The fast dissociation rate of the *p*-nitrophenyl phosphate-supported complex is observed in spite of a much higher ouabain binding here than with P_i (Table I). In a parallel experiment the dissociation of the (Na^+ + Mg^{2+} + *p*-nitrophenyl-*P*)-

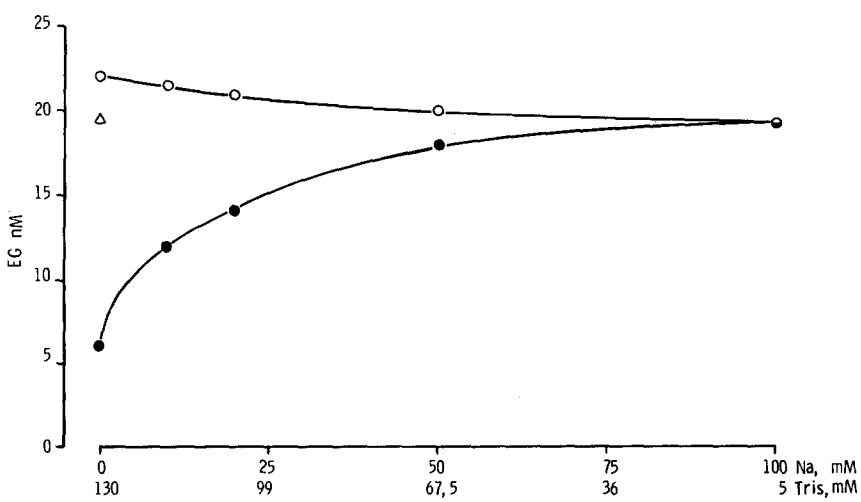

Fig. 5. Effect of the concentration of Na^+ on the ouabain equilibrium binding level (EG) obtained with (Mg^{2+} + *p*-nitrophenyl-*P*) at an increasing (○) or fixed ionic strength (●). Enzyme (0.19 mg protein/ml) was incubated for 180—240 min at 37°C with 5 mM Mg^{2+}, 1 mM *p*-nitrophenyl phosphate, 0—100 mM Na^+, $2.5 \cdot 10^{-8}$ M [^{3}H]ouabain and 5 mM Tris (○) or 5—130 mM Tris (●), without Na^+ also 35 mM Tris (△).

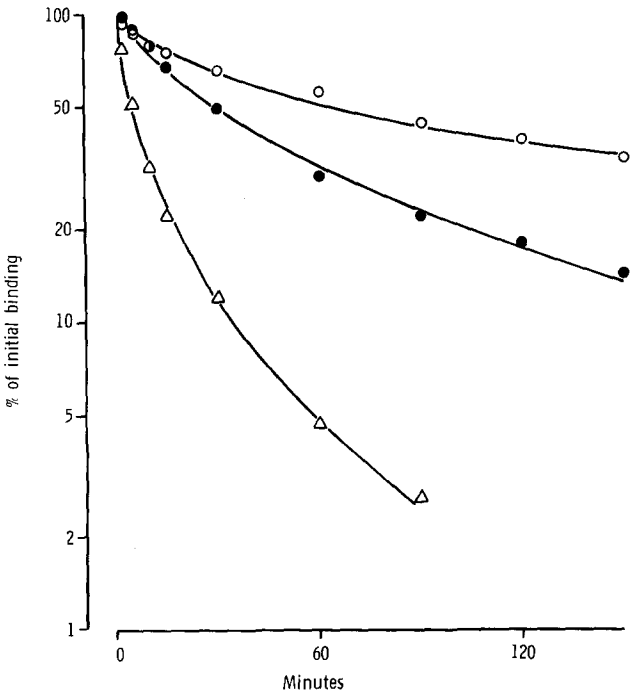

Fig. 6. Ouabain dissociation rates after (Mg^{2+} + *p*-nitrophenyl phosphate)-facilitated ouabain binding with 100 mM Na^+ or K^+ present during binding. 0.19 mg protein/ml (ouabain-sensitive activity 202 μmol P_i/mg protein per h) was incubated for 1 h with 3 mM Mg^{2+}, 1 mM *p*-nitrophenyl phosphate, 5 mM Tris (pH 7.25), $2 \cdot 10^{-7}$ M [^{3}H]ouabain (△) and (△○), 100 mM Na^+ or ●, 100 mM K^+. Incubation was terminated and the enzyme · ouabain complex isolated at 0°C as described in Materials and Methods. The yield after incubation with Na^+ 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^+ (○). The enzyme · ouabain complex obtained with K^+ was resuspended in 4 mM Tris. In all cases 10^{-3} M ouabain was present. The release of [^{3}H]ouabain at 37°C was determined as described and expressed as percent of initial binding after wash and resuspension at 0°C.

facilitated complex took place in the presence of 2 mM K^+ and it is seen from Fig. 6 that the rate of decay is very much retarded. In Fig. 6 is also shown the dissociation of enzyme · ouabain complex in Tris buffer after (Mg^{2+} + *p*-nitrophenyl-*P*)-supported ouabain binding in the presence of 100 mM K^+. It is seen that the dissociation rate in Tris, after binding in the presence of K^+, is faster than when binding occurred in the presence of Tris (compare with the lower curve in Fig. 2).

To ascertain whether the dissociation rate, unlike the binding level, was independent of the ionic strength or possibly the Tris ion as such during binding, the rates of decay of the complex obtained in the presence of (Mg^{2+} + *p*-nitrophenyl-*P*) and 5 mM Tris or 130 mM Tris were compared. Although 367 and 47 pmol ouabain were bound per mg protein, respectively, the dissociation rates were the same in the two situations and identical with those shown in Fig. 2.

Discussion

Cardiac glycosides (e.g. ouabain) are specific inhibitors of the $(Na^+ + K^+)$-activated ATPase, and are firmly bound to the enzyme under a variety of conditions but with an apparent affinity which varies with the facilitating ligands employed for binding [4]. Enzyme · ouabain complexes formed under various conditions can also be characterized by their dissociation rates [12,13] as observed after removal of the facilitating ligands. Ouabain binding rates as well as enzyme-ouabain dissociation rates are slow and, therefore, amenable to accurate measurements. Ouabain binding under different binding conditions and characterization of the enzyme · ouabain complexes by their dissociation properties may thus be looked upon as methods for stabilizing and analysing otherwise transitional enzymatic states created by the ligands.

From the present observations two conclusions may be drawn. (1) The sharp distinction [12,13] between the $(Mg^{2+} + Na^+ + ATP)$-supported enzyme · ouabain complex and the $(Mg^{2+} + P_i)$- and $(Mg^{2+} + p$-nitrophenyl-$P)$-supported complexes seems arbitrary since the latter two can be converted to the former complex in the presence of a high concentration of Na^+ during binding. (2) The monovalent cation present during ouabain binding determines the character of the enzyme · ouabain complex, an observation which emphasizes that Na^+, K^+ and possibly the Tris ion per se affect the enzyme [15].

The enzyme · ouabain complexes obtained with the 'substrates' p-nitrophenyl phosphate, P_i and probably also ATP in the presence of the same monovalent cation seem qualitatively to be the same judging from their dissociation properties. On the other hand, monovalent cations and especially Na^+ greatly influence the properties of the enzyme · ouabain complex. The differences reported previously [6,10,12,13] are mainly due to the presence or absence of Na^+ during binding. With omission of Na^+ and in the presence of only Tris ions the $(Mg^{2+} + p$-nitrophenyl-$P)$- and $(Mg^{2+} + P_i)$-supported enzyme · ouabain complexes seem to be identical and insensitive to K^+. At high Na^+ concentration during binding the complexes in both cases acquire characters very similar to those attributed to the $(Mg^{2+} + Na^+ + ATP)$-supported complex [12,13]. The dissociation becomes fast and K^+ sensitive. A slow ouabain binding also takes place with just Mg^{2+} plus ATP, and this complex shows a slower decay than that formed in the presence of Na^+ too [19].

Besides the Na^+-induced form of the enzyme · ouabain complex a distinct K^+-induced form is obtained in the presence of K^+ during formation of the complex. The most inert form is obtained in the presence of Tris ions or possibly in the absence of Na^+ as well as K^+. With graded trypsin digestion of the catalytic protein of $(Na^+ + K^+)$-ATPase Jørgensen [15] was able to identify a K-form and a Na-form of the enzyme in the presence of the respective ions. The Na-form was converted to the K-form by $Mg^{2+} + Na^+ + ATP$. The present observations suggest that transitions between Na- and K-forms are also expressed in altered reactivity of the ouabain binding sites. The explanation of the fast decay in Tris buffer of the complexes obtained in the presence of Na^+ and their K^+ sensitivity during dissociation might be the following. Phosphorylation obviously takes place in the presence of $(Mg^{2+} + Na^+ + ATP)$ and possibly also in the presence of $(Mg^{2+} + Na^+ + P_i)$ and $(Mg^{2+} + Na^+ + p$-nitrophenyl-$P)$ to

a similar high energy phosphate form. Thus during binding of ouabain the K-form of the enzyme is prevalent under these conditions. During the dissociation in Tris buffer only, dephosphorylation of the enzyme · ouabain complex may already have taken place according to the observation of Sen et al. [20]. The dephosphoenzyme then relaxes to the Na-form, which does not retain ouabain. On addition of K^+ during dissociation the enzyme · ouabain complex is fixed in the K-form and ouabain dissociation is retarded. The explanation also implies that ouabain binding in the presence of (Mg^{2+} + P_i) or (Mg^{2+} + *p*-nitrophenyl-*P*) takes place to the K-form of the enzyme.

Comparison of the present observations with those obtained in phosphorylation experiments is also tempting. (Na^+ + K^+)-ATPase can be phosphorylated from ATP as well as from P_i [21], and the two phosphorylated complexes are interconvertible since ATP can be produced from the phosphoenzyme formed from inorganic phosphate in the presence of ADP and a high concentration of Na^+ [22]. Whether phosphorylation is a prerequisite for ouabain binding, is an unanswered question, the main objection being that some degree of binding takes place even under conditions where phosphorylation ought to be absent, e.g. with only Mg^{2+} present. However, (Mg^{2+} + P_i)-supported ouabain binding to (Na^+ + K^+)-ATPase [4] and the effects of K^+ and Na^+ on the binding have a striking likeness to observations with phosphorylation of the enzyme with P_i [21] and to studies of ^{18}O exchange between water and P_i [23]. Furthermore, the enzyme · ouabain complex obtained with only (Mg^{2+} + P_i) and characterized by a slow and K^+-insensitive rate of dissociation resembles the insensitive phosphoenzyme obtained under these conditions [22]. Sen et al. [20] have shown that upon exposure of ATP-derived phosphoenzyme to ouabain the drug was bound, but on the other hand they also found evidence of a slow dephosphorylation of the enzyme · ouabain complex and that rephosphorylation with P_i, but not with ATP was possible. Whether permanently phosphorylated or not one could imagine that the reactive state of the enzyme during ouabain binding was retained to define the dissociation character of the enzyme · ouabain complex. The situations giving rise to enzyme · ouabain complexes with fast and K^+-sensitive dissociation rates seem identical with those which give rise to K^+-sensitive phosphoenzyme formed from P_i [21]. Such phosphoenzyme is able to phosphorylate ADP.

Due to the ADP : ATP phosphotransferase activity of (Na^+ + K^+)-ATPase one might expect a transfer of ^{32}P from *p*-nitrophenyl[^{32}]phosphate to ADP to produce [^{32}P]ATP under the conditions of formation of the *p*-nitrophenyl phosphate-supported ouabain complex with the fast dissociation rate. Hobbs and De Weer [24] found no evidence of this process at a low Mg^{2+} concentration and more remarkably, neither did transfer of ^{32}P from [^{32}P]ATP to *p*-nitrophenol take place under conditions where different phosphoenzyme intermediates were supposed to be present. Experiments are in progress to examine whether the former process is detectable at a higher Mg^{2+} concentration.

With K^+ plus Na^+, ATP increases the K^+-activated *p*-nitrophenylphosphatase activity when the K^+ : Na^+ ratio is low, but moreover even without ATP, a high concentration of Na^+ increases the activity in the presence of a low concentration of K^+ (Fig. 7 of ref. 25). An explanation consistent with this observation and with the identical kinetic behaviour of the ouabain binding states obtained

with different substrates at high Na^+ concentration would be that the same phosphorylated state of the enzyme is obtained and that this state (possibly dephosphorylated, but retaining the reactive state) accepts *p*-nitrophenyl phosphate as substrate leading to an increased K^+-activated *p*-nitrophenyl phosphate hydrolysis.

Most of the ouabain dissociation curves are definitely not monoexponential. One may wonder whether the two component dissociation curves obtained after (Mg^{2+} + P_i)-supported ouabain binding with Tris buffer and the "nearly monoexponential" curve with K^+ added during binding is compatible with the observed two (or more) populations of ouabain binding sites in the former situation and the apparent conversion to uniformity by K^+ as described earlier [7].

Acknowledgements

The skilled technical assistance of Ms. Kirsten Pedersen is gratefully acknowledged. Thanks are due to Professor J.C. Skou for helpful discussions during preparation of the manuscript.

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