Biochimica et Biophysica Acta, 330 (1973) 302–315

BBA 76508

OUABAIN–RECEPTOR INTERACTIONS IN $(Na^+ + K^+)$-ATPase PREPARATIONS

II. EFFECT OF CATIONS AND NUCLEOTIDES ON RATE CONSTANTS AND DISSOCIATION CONSTANTS

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(Received July 23rd, 1973)

SUMMARY

The action of ATP and its analogs as well as the effects of alkali ions were studied in their action on the ouabain receptor. One single ouabain receptor with a dissociation constant (K_D) of 13 nM was found in the presence of $(Mg^{2+} + P_i)$ and $(Na^+ + Mg^{2+} + ATP)$. pH changes below pH 7.4 did not affect the ouabain receptor. Ouabain binding required Mg^{2+}, where a curved line in the Scatchard plot appeared. The affinity of the receptor for ouabain was decreased by K^+ and its congeners, by Na^+ in the presence of $(Mg^{2+} + P_i)$, and by ATP analogs (ADP–C–P, ATP–OCH_3). Ca^{2+} antagonized the action of K^+ on ouabain binding. It was concluded that the ouabain receptor exists in a low affinity (R_α) and a high affinity conformational state (R_β). The equilibrium between both states is influenced by ligands of $(Na^+ + K^+)$-ATPase. With 3 mM Mg^{2+} a mixture between both conformational states is assumed to exist (curved line in the Scatchard plot).

INTRODUCTION

Ouabain is a specific and powerful inhibitor of the sodium pump[1–3] and the $(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, E.C. 3.6.1.3) of cell membranes[4] for reviews see[5–9]. This inhibitory action on $(Na^+ + K^+)$-activated ATPase may very well be correlated to the positive inotropic action of ouabain on heart muscle[10–12]. Because of this assumption the interaction of cardiac glycosides with its receptor has been investigated in detail by many research groups[12–27]. It was demonstrated that the binding capacity of cell membranes for [^{3}H]ouabain is linearly related to the $(Na^+ + K^+)$-ATPase activity[14,18,28]. In addition, much evidence has been accumulated that the specific binding of [^{3}H]ouabain to $(Na^+ + K^+)$-ATPase preparations from different tissues runs parallel to the inhibition of the enzyme system[14,16,23,29–31]. Binding of [^{3}H]ouabain (O) to its receptor (R) is a reversible process and can be described by the mass law equation (Eqn 1)[18,28,32–34]:

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$$O + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} OR \quad (1)$$

The dissociation constant of the ouabain–receptor complex has been determinded in a previous publication[28] for various species and tissues under specified conditions (*i.e.* 3 mM Mg^{2+} + 3 mM P_i). Ouabain binding, however, is considerably influenced by the presence of substrate and products of $(Na^+ + K^+)$-ATPase and by univalent cations[4,5,23,29,22]: An Na^+-dependent and an Na^+-independent pathway of ouabain binding has been reported[22]. ATP, which is necessary for the Na^+-dependent pathway, can be replaced by other nucleotide triphosphates[35]. In contrast to this finding a mutual exclusion of ATP, ADP and ouabain has been reported[36]. Similar discrepancies are evident from the literature with respect to the effects of K^+ on ouabain binding: K^+ has been reported to slow down the binding process[12,14,16,17,37] or to retard the dissociation of the glycoside from its receptor[17,38,39,40]. In addition two ouabain-binding sites with different affinities have been reported. According to Taniguchi and Iida[33] absence of K^+ decreases the number of low affinity sites by approximately one half.

In order to clarify these contradictions the effects of nucleotides and cations were measured in terms of association and dissociation rate constants as well as of dissociation constants. This paper shows that all changes in ouabain binding are due to variations of the dissociation constant of the ouabain–receptor complex but not due to variations of the maximal number of binding sites.

METHODS AND MATERIALS

Preparation and quantitation of enzyme

$(Na^+ + K^+)$-ATPase (E.C. 3.6.1.3) from beef brain was prepared as described previously[41]. The enzyme preparation from beef kidney was isolated according to Post and Sen[42] with the modification that heparin was not used, $(Na^+ + K^+)$-ATPase activity was measured with the coupled optical assay[41]. The activity was continuously recorded and corrected for Mg^{2+}-activated ATPase by inhibition of $(Na^+ + K^+)$-ATPase with 10^{-3} M ouabain. ATP was converted to the free acid by passage through Dowex (H^+ form) and neutralized with tris(hydroxymethyl)-aminomethane.

One enzyme unit is defined as the amount of enzyme hydrolyzing 1 μmole ATP per min at 37 °C. Protein was quantitated by the procedure of Lowry *et al.*[43]. The assay of [^{3}H]ouabain binding and [^{3}H]ouabain release by a rapid centrifugation method is described in great detail in a previous paper in this journal[28]. The same is true for the $(Na^+ + K^+)$-ATPase assay.

[^3H]Ouabain binding under equilibrium conditions

The time needed for equilibrium binding was determined in preliminary experiments with the lowest possible glycoside concentrations (Fig. 1) = $7.5 \cdot 10^{-9}$ M of undiluted [^{3}H]ouabain. Successive experiments were carried out with incubation periods of 60 min or more at 37 °C. When cation effects were studied, the incubation time was accordingly extended.

Unless otherwise indicated the incubation medium consisted of 50 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, 2 mM Tris–phosphate, various amounts of

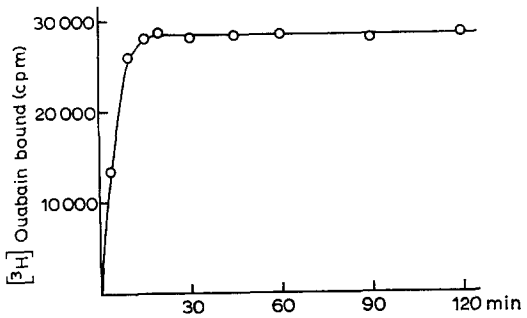

Fig. 1. Determination of equilibrium of ouabain binding to $(Na^+ + K^+)$-ATPase. 0.3 mg enzyme protein (specific activity 3.3 units/mg protein, ox brain) were incubated in 50 mM imidazole–HCl pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate and $7.5 \cdot 10^{-9}$ M [^{3}H]ouabain at 37 °C for the indicated time.

unlabeled ouabain in addition to $7.5 \cdot 10^{-9}$ M [^{3}H]ouabain and 0.2–0.4 mg of enzyme protein. The total volume was usually 2 ml. It should be mentioned that higher amounts of protein in the same volume resulted in a curved line in the Scatchard plot probably due to mixing difficulties during the incubation period. This could easily be avoided by taking low amounts of enzyme protein. All experiments were performed in duplicate or triplicate assays. Specific [^{3}H]ouabain binding is obtained by subtracting from the total radioactive uptake the amount that is not displaced by high concentrations (10^{-4} M) of unlabeled ouabain.

MATERIALS

[^{3}H]Ouabain with a specific activity of 13 Ci/mmole was obtained from New England Nuclear, Dreieichenhain, Germany. All other chemicals were of analytical grade and obtained through Boehringer, Mannheim or E. Merck AG, Darmstadt.

Adenyl-diphosphonate-(α,β-methylene), AMP–C–P–P and adenyldiphosphonate-(β,γ-methylene), ADP–CP were from Serva, Heidelberg. ATP-γ-S, the methyl ester of ATP (ATP–OCH_3) and [^{3}H]ATP-γ-S (spec. act. $15 \cdot 10^6$ cpm/μmole) were kind gifts of Dr Fritz Eckstein, Max-Planck-Institut für Experimentelle Medizin, D-34-Göttingen, Germany.

RESULTS

[^{3}H]Ouabain has a weak affinity for $(Na^+ + K^+)$-ATPase preparations (Table I). However, the presence of 0.5 to 10 mM Mg^{2+} or Mn^{2+} considerably increases the affinity of the ouabain receptor towards the cardiac glycoside (Table I). In the presence of Mn^{2+} or Mg^{2+} at least two ouabain binding sites with slightly different dissociation constants are seen in beef brain (Fig. 2), but not in beef kidney (not shown). In contrast to this result only one binding site with higher affinity for ouabain is found in the presence of $Mg^{2+} + P_i$. The addition of P_i decreases the average K_D of ouabain in the presence of Mg^{2+} by about four times (Table I).

It is interesting to see that changes in pH values below pH 7.4 do not show

TABLE I

EFFECT OF SUBSTRATE, SUBSTRATE ANALOGS AND PRODUCTS OF $(Na^+ + K^+)$-ATPase ON THE DISSOCIATION CONSTANT (K_D) OF THE OUABAIN-RECEPTOR-COMPLEX

0.2–0.4 mg enzyme protein were incubated at 37 °C in 50 mM imidazole–HCl, pH 7.25, and varying amounts of [^{3}H]ouabain. The dissociation constants are calculated from Scatchard plots.

Experimental conditions	K_D (nM)
Kidney enzyme	
No additions	2600
1–10 mM Mg^{2+}	39
3 mM Mg^{2+} + 0.5 mM P_i	13
3 mM Mg^{2+} + 10 mM P_i	13
Brain enzyme	
3 mM Mn^{2+}	48 *
3 mM Mg^{2+} + 3 mM P_i	12.5
3 mM Mg^{2+} + 3 mM ATP	41
3 mM Mg^{2+} + 3 mM ATP + 100 mM Na^+	13.5
3 mM Mg^{2+} + 3 mM ADP	262
3 mM Mg^{2+} + 3 mM ADP + 100 mM Na^+	35
3 mM Mg^{2+} + 3 mM ADP-C-P	240
3 mM Mg^{2+} + 3 mM ADP-C-P + 100 mM Na^+	— **
3 mM Mg^{2+} + 3 mM AMP-C-P-P	935
3 mM Mg^{2+} + 3 mM AMP-C-P-P + 100 mM Na^+	54
3 mM Mg^{2+} + 4 mM ATP-γ-S	36
3 mM Mg^{2+} + 4 mM ATP-γ-S + 100 mM Na^+	10
3 mM Mg^{2+} + 3 mM ATP-OCH_3	— ***
2 mM Mg^{2+} + 3 mM ATP-OCH_3 + 100 mM Na^+	— **

* Average value.
** No binding of [^{3}H]ouabain.
*** Very small amount of binding of [^{3}H]ouabain.

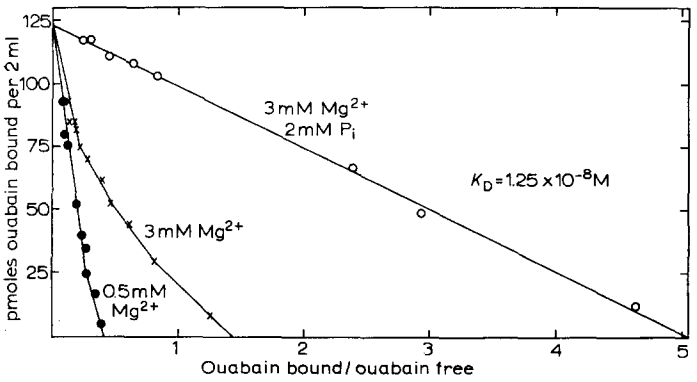

Fig. 2. Ouabain binding in the presence of Mg^{2+}. 0.27 mg protein (ox brain, $(Na^+ + K^+)$-ATPase activity 3.5 units/mg protein) were incubated for 120 min at 37 °C in 50 mM imidazole–HCl pH 7.25, 0.5 mM and 3 mM $MgCl_2$ (±2 mM Tris–phosphate) and increasing amounts of [^{3}H]-ouabain. Total volume 2 ml. ○—○, in the presence of 3 mM Mg^{2+} + 2 mM P_i; ×—×, in the presence of 3 mM Mg^{2+}; ●—●, in the presence of 0.5 mM Mg^{2+}.

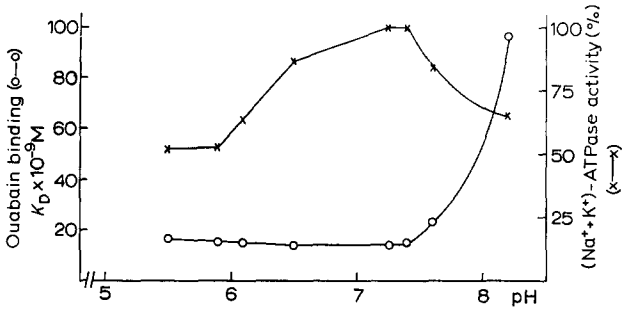

Fig. 3. Dependence of the $(Na^+ + K^+)$-ATPase activity and the dissociation constant of the ouabain–receptor complex on pH. $(Na^+ + K^+)$-ATPase activity was measured in the optical coupled assay[41]. The dissociation constants were calculated from Scatchard plots[52]; 0.23 mg of protein (ox brain, specific activity 2.7 units/mg protein) were incubated for 40 min in 50 mM imidazole–HCl buffer or 50 mM Tris–HCl buffer (pH 8.2), 3 mM $MgCl_2$, 3 mM Tris–phosphate, increasing amounts of [^{3}H]ouabain at 37 °C, total volume 2 ml. Symbols: ×—×, $(Na^+ + K^+)$-ATPase activity; ○—○, ouabain binding.

any changes in the affinity of the ouabain–receptor system (Fig. 3). This is in contrast to the effect of pH on the overall-reaction of $(Na^+ + K^+)$-activated ATPase. Alkalization of the medium above pH 7.4, however, induces a considerable change of the dissociation constant of the ouabain–receptor complex. A similar pattern of a plot of the dissociation constant *versus* pH is found for the ATP–enzyme complex[37] and ADP–enzyme complex[44] of $(Na^+ + K^+)$-activated ATPase. Binding of ouabain occurs at the outside of the cell membrane and binding of nucleotides at the inside, therefore it appears plausible to assume, that the considerable increase in the dissociation constant of the ouabain–receptor and the nucleotide–receptor complex indicates an alteration of the cell membrane structure at alkaline pH. The same interpretation should be valid for the decrease of the velocity of ATP hydrolysis by $(Na^+ + K^+)$-activated ATPase above the pH optimum (Fig. 3).

Inorganic phosphate, which decreases the dissociation constant of the ouabain–receptor complex cannot be substituted by ADP, the second product of $(Na^+ + K^+)$-activated ATP hydrolysis, or any other nucleotide (Table I). On the contrary ADP and the ATP analogs ADP–C–P, AMP–C–P–P and ATP–OCH_3 increased the dissociation constant. Binding of ADP–C–P and AMP–C–P–P to the nucleotide binding site of $(Na^+ + K^+)$-ATPase has been demonstrated[37]. ATP-γ-S and ATP–OCH_3 bind to $(Na^+ + K^+)$-ATPase too, as is evident from the competition with [^{14}C]ADP for the nucleotide site of the enzyme (Fig. 4A). But in contrast to ATP, the terminal phosphate is not split off from ADP–C–P and ATP–OCH_3 by the enzyme. The hydrolytic activity of the enzyme with ATP-γ-S is about 1/2500 of that with ATP; but the enzyme is inhibited by K^+ (Fig. 4B). A similar finding has been reported for the hydrolysis of UTP by $(Na^+ + K^+)$-ATPase[60]. Nevertheless the decrease of the dissociation constant for ouabain in the presence of 100 mM Na^+ with ATP-γ-S as substrate reaches the same value as with ATP (10 nM) (Table I). Similar observations in the presence of 100 mM Na^+ were made with AMP–C–P–P and ADP, but not with ATP–OCH_3 and ADP–C–P. As already mentioned ADP–C–P and ATP–OCH_3 is not a substrate of the $(Na^+ + K^+)$-ATPase, but AMP–C–P–P can be hydrolyzed. The stimulatory action of Na^+ on ouabain binding in the presence

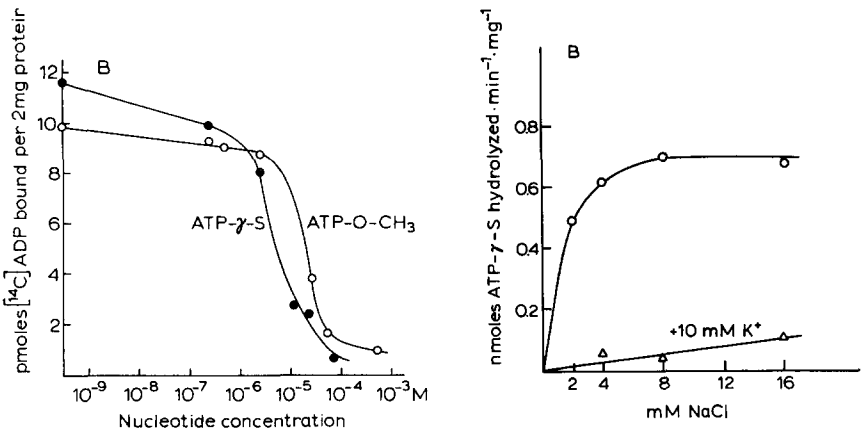

Fig. 4. (A) Affinity of ATP-γ-S and ATP–O–CH_3 to the ADP-binding site of $(Na^+ + K^+)$-ATPase. 2 mg enzyme protein were incubated for 1 min at 0 °C with $93 \cdot 10^{-12}$ M [^{14}C]ADP and increasing amounts of unlabelled ATP-γ-S and ATP–O–CH_3. The radioactive ADP not displaced by the unlabelled nucleotides was measured in the $80000 \times g$ sediment. (B) Hydrolysis of [^{3}H]ATP-γ-S by $(Na^+ + K^+)$-activated ATPase. 27 μg $(Na^+ + K^+)$-ATPase (specific enzymatic activity: 1.8 μmoles·min^{-1}·mg^{-1}) was incubated at 37 °C for 60 min in 40 mM imidazole, pH 7.25, 2.3 mM [^{3}H]ATP-γ-S, 6 mM $MgCl_2$ and the additions shown. 5 μl of the reaction mixture was given on thin-layer chromatography plastic sheets PEI-cellulose F (Merck AG). ATP-γ-S was separated from the reaction products by chromatography in 0.75 M K_2HPO_4, pH 3.5. The [^{3}H]ATP-γ-S spots were cut out and counted. Each point represents average values of experiments done in triplicate.

of ADP is most probably due to contamination of the membrane preparation with adenylate kinase activity, because an impurity of 12 munits/mg protein was repeatedly found in $(Na^+ + K^+)$-ATPase preparations. ATP formed by the disproportion of 2 moles ADP by adenylate kinase is very probably the substrate for the well known Na^+-dependent phosphorylation reaction and the Na^+-stimulated binding of ouabain. In accordance with this assumption, concentrations of 3 mM Na^+ decreased the dissociation constant of the ouabain–receptor complex half-maximally in the presence of ADP (Fig. 5). Much higher concentrations of Na^+ (20 mM) were necessary to produce half-maximal decrease of K_D values in the presence of ATP. It is interesting to see, that low concentrations of Na^+ increase the dissociation constant, whenever an Na^+-dependent phosphorylation is not possible, *i.e.* in the presence of Mg^{2+} alone (Fig. 5) or $Mg^{2+} + P_i$ (not shown). Since it is not possible to form a phosphorylated intermediate with the nucleotides ADP–C–P and ATP–OCH_3, Na^+ even further impairs the affinity of the receptor towards ouabain, although it is already low in the presence of Mg^{2+} *plus* these nucleotides (Table I).

Effect of K^+ and its congeners on ouabain binding

The results presented above indicate, that Mg^{2+}, Na^+ and nucleotides alter the conformational state of the ouabain receptor. A decrease of [^{3}H]ouabain binding by K^+ has often been reported[5,12,14,16,39,40,45]. Nevertheless, it is still quite controversial as to whether K^+ slows the binding[12,14,16,17,39] and/or retards the dissociation of ouabain from its receptor[17,38–40].

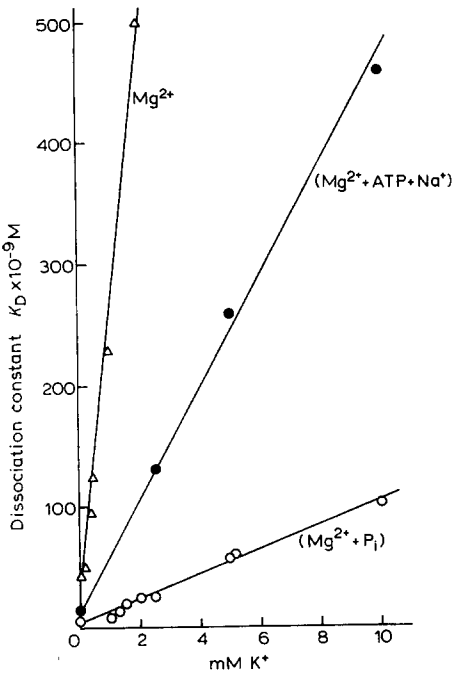

Fig. 5. Na^+-effect on the dissociation constant (K_D) of the ouabain–receptor complex. 0.34 mg of enzyme protein (ox brain specific activity 2.0 units/mg protein) were incubated in 50 mM imidazole–HCl, pH 7.2, 3 mM $MgCl_2$ and either 3 mM Tris–ATP or 3 mM Tris–ADP, increasing amounts of [^{3}H]ouabain and increasing amounts of Na^+ for 40 min (ATP), 80 min (ADP and Mg^{2+}) until equilibrium was reached. Total volume 2 ml, 37 °C. The dissociation constants are calculated from Scatchard plots[52].

In addition Taniguchi and Iida[33] reported that K^+ affects the number of ouabain-binding sites with low affinity. As is evident from Fig. 6 only one single high affinity ouabain-receptor site is found in $(Na^+ + K^+)$-ATPase from ox brain with 3 mM Mg^{2+} + 3 mM P_i. As can be seen from the Scatchard plot, K^+ and its congeners increase the dissociation constant of the ouabain–receptor complex, but the number of binding sites is not altered. A quantitative analysis of these cation effects on [^{3}H]ouabain binding in the presence of $Mg^{2+} + P_i$ is presented in Table II: In agreement with Schönfeld *et al.*[14] the apparent affinity of univalent cations for the ouabain receptor is: $K^+ \geqq Rb^+ > NH_4^+ > Cs^+ > Na^+ > Li^+$.

The studies of Nørby and Jensen[46] and of Hegyvary and Post[37] on the effects of K^+ on ATP binding showed that the nucleotide receptor was affected by very low K^+ concentrations. Maximal alterations were already obtained with 1 mM K^+ (ref. 46). In contrast to the effect of K^+ on the nucleotide receptor, potassium decreased the affinity of the receptor for ouabain almost linearly with the K^+ concentration. This effect is more pronounced in the presence of Mg^{2+} or $(Mg^{2+} + ATP + Na^+)$ than with $(Mg^{2+} + P_i)$ (Fig. 7). Measurements of the dissociation rate constant in the presence of 3 mM Mg^{2+}, 3 mM Tris–phosphate and 5 mM K^+ revealed an unchanged dissociation rate constant ($k_{-1} = 1.27 \cdot 10^{-4}\ s^{-1}$) as compared

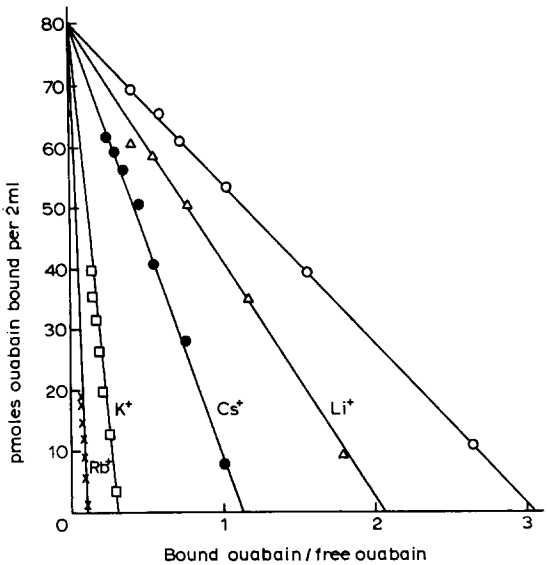

Fig. 6. Scatchard plots of ouabain binding in the presence of monovalent cations. 0.3 mg of enzyme protein (ox brain, specific activity 2.05 units/mg protein= $266 \cdot 10^{-12}$ moles receptor sites per mg protein) were incubated in 50 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate and increasing amounts of labelled ouabain at 37 °C for 120 min. Total volume 2 ml. Addition of 20 mM RbCl (×—×), 10 mM KCl (□—□), 20 mM CsCl (●—●), 20 mM LiCl (△—△), without any addition ○—○. The dissociation constant is calculated from the slop of the plot.

to a k_{-1} of $1.22 \pm 0.2 \cdot 10^{-4}\ s^{-1}$ without K^+ (ref. 28) (both experiments were performed with ox brain $(Na^+ + K^+)$-ATPase preparations at 37 °C) (Fig. 8B). The association rate constant, however, is decreased from $2.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ to $0.21 \cdot 10^{-4}\ M^{-1} \cdot s^{-1}$ by 5 mM K^+ in the presence of $(Mg^{2+} + P_i)$ (Fig. 8A). The same conditions were found with $(Na^+ + K^+)$-ATPase preparations from other tissues or in the presence of $(Mg^{2+} + ATP + Na^+)$.

TABLE II

EFFECTS OF ALKALI IONS ON THE DISSOCIATION CONSTANT OF OUABAIN-RECEPTOR COMPLEX OF $(Na^+ + K^+)$-ATPase FROM BEEF BRAIN IN THE PRESENCE OF 3 mM Mg^{2+} + 3 mM P_i

20 mM Effector	K_D (*nM*)
Li^+	18.8
Na^+	28.0
K^+	205
NH_4^+	82.5
Rb^+	197.5
Cs^+	34.6

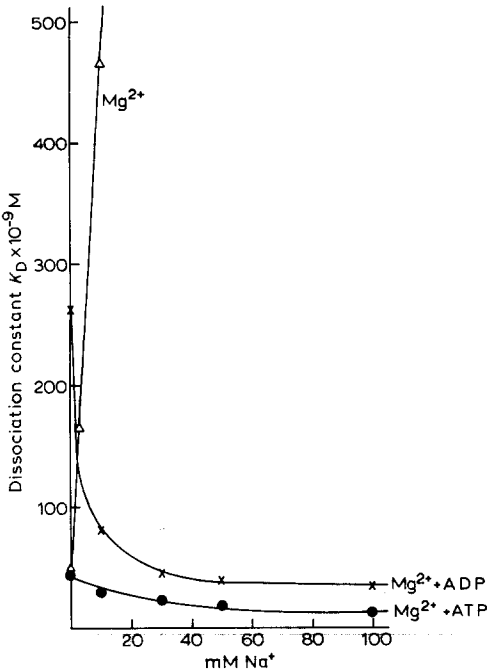

Fig. 7. Dependence of the dissociation constant of the ouabain–receptor complex on the potassium concentration. The dissociation constants were calculated from Scatchard plots[52]: 0.76 mg of enzyme protein (beef kidney, specific activity 0.71 units/mg protein) were incubated in 50 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate or 3 mM Tris–ATP+80 mM NaCl and increasing amounts of [^{3}H]ouabain until equilibrium was reached (up to 120 min with 10 mM K^+) at 37 °C. Total volume 2 ml. ○—○, $Mg^{2+}+P_i$; ●—●, $Mg^{2+}+ATP+Na^+$; △—△ 3 mM Mg^{2+} alone.

Effect of Na^+ on the interaction of K^+ with the ouabain-receptor site

K^+ decreases the affinity of nucleotides[37,46,47] and of ouabain for their binding sites. This action of K^+ on nucleotide binding is abolished by Na^+ (refs 37, 47). In order to see, whether a similar effect of Na^+ is seen on the ouabain receptor, the effect of increasing Na^+ concentrations on the dissociation constant of ouabain–receptor complex at 5 mM K^+ was investigated. As is evident from Fig. 9, Na^+ shows two different kinds of actions on ouabain binding: Na^+ in the presence of ($Mg^{2+}+ATP+K^+$) decreases the affinity of the ouabain receptor at low concentrations while it increases the affinity at high concentrations.

The effect of Na^+ above 30 mM is probably due to a competition with K^+ for a common site[48]. The reason for the increase of the dissociation constant at low Na^+ concentrations in the presence of K^+ remains to be clarified.

Effect of Mg^{2+} concentration on the interaction of Na^+ and K^+ with the ouabain receptor

A comparision of the Na^+ and/or K^+ concentrations necessary to reduce [^{3}H]ouabain binding with those necessary to affect nucleotide binding to the same enzyme shows that considerably higher concentrations of K^+ and Na^+ are necessary

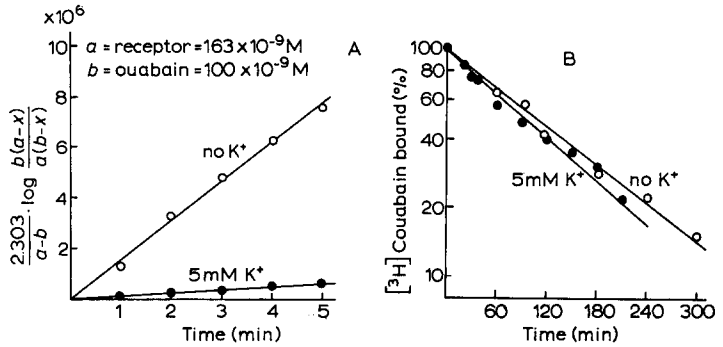

Fig. 8. Dependence of the rates of association (k_{+1}) and dissociation k_{-1} on the potassium concentrations. (A) 1 mg of enzyme protein (ox brain, specific activity 2.51 units/mg protein= $326 \cdot 10^{-12}$ moles receptor sites) were incubated in 50 mM imidazole-HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris-phosphate (± 5 mM KCl) and $100 \cdot 10^{-9}$ M [^{3}H]ouabain at 37 °C for the indicated time. Total volume 2 ml. Initial receptor concentration, $a = 163 \cdot 10^{-9}$ M. Initial ouabain concentration, $b = 100 \cdot 10^{-9}$ M. At the indicated time the reaction was stopped by rapid freezing in liquid air and centrifugation at $80000 \times g$ for 30 min at 0 °C. Radioactivity in the sediment was assayed as previously reported[28]. k_{+1} is calculated according to the equation for bimolecular reactions:

$$k_{+1} = \frac{2.303}{(a-b)} \cdot \log \frac{b(a-x)}{a(b-x)}$$

k_{+1} without K^+, $2.5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$; k_{+1} with 5 mM K^+, $0.21 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. (B) 20 mg of enzyme protein (same as in A) were incubated in 100 mM imidazole-HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris-phosphate and $0.4 \cdot 10^{-6}$ M [^{3}H]ouabain at 37 °C for 60 min. After washing this labelled enzyme-ouabain complex twice at 0 °C in 0.01 M imidazole-HCl, pH 6.5, in the ultracentrifuge ($80000 \times g$) to remove the unbound labelled ouabain, the protein was taken up in 0.01 M imidazole-HCl, pH 6.5. 0.4 mg of this labelled enzyme-ouabain complex was incubated in 50 mM imidazole-HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris-phosphate (± 5 mM KCl) and 10^{-4} M unlabelled ouabain for the indicated time. Total volume 2 ml. The reactions was stopped and the amount of enzyme-[^{3}H]ouabain complex was determined as in A: k_{-1} without K^+ $1.27 \cdot 10^{-4}$ s^{-1}. k_{-1} with 5 mM K^+, $1.22 \cdot 10^{-4}$ s^{-1}.

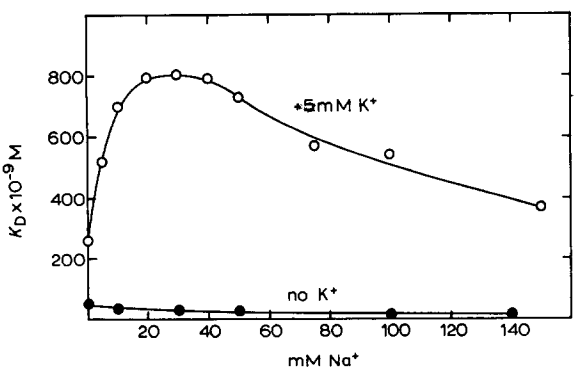

Fig. 9. Effect of Na^+ on the dissociation constant (K_D) of the ouabain-receptor complex with and without K^+. 0.2 mg protein (ox brain, $(Na^+ + K^+)$-ATPase activity= 3.3 units/mg protein) were incubated for 120 min in 50 mM imidazole-HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM ATP, (5 mM KCl) increasing amounts of [^{3}H]ouabain and varying NaCl concentrations at 37 °C. Total volume 2 ml. The dissociation constants are calculated from Scatchard plots. ○—○, with 5 mM KCl; ●—●, without KCl.

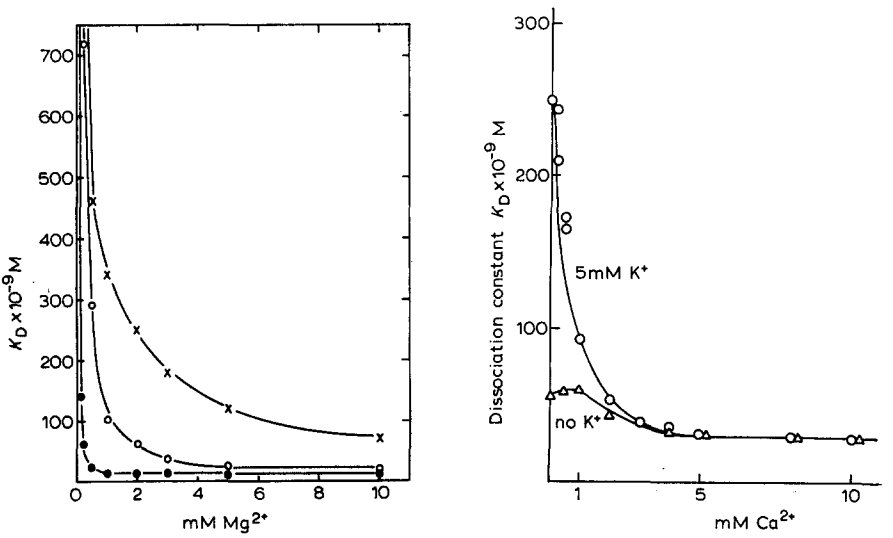

Fig. 10. Effect of Mg^{2+} concentration on the increase of K_D values by K^+ and Na^+. 0.35 mg of enzyme protein (ox brain, $(Na^+ + K^+)$-ATPase activity 3.0 units/mg protein) were incubated for 120–180 min in 50 mM imidazole–HCl, pH 7.25, 3 mM imidazole–phosphate and increasing amounts of [^{3}H]ouabain. Total volume 2 ml. The dissociation constants were calculated from Scatchard plots[52]. ●—●, no further additions but $MgCl_2$; ○—○, in the presence of 20 mM NaCl; ×—×, in the presence of 5 mM KCl.

Fig. 11. Dependence of the dissociation constant of the ouabain–receptor complex on the Ca^{2+} concentrations. 0.2 mg of enzyme protein (ox brain, specific activity 3.4 units/mg protein) were incubated in 50 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–ATP (±5 mM KCl) and increasing amounts of [^{3}H]ouabain at 37 °C until equilibrium was reached (100 min). Total volume 2 ml. The dissociation constants were calculated from Scatchard plots[52]. ○—○, in the presence of 5 mM K^+; △—△, without K^+.

to demonstrate changes of the K_D value of the ouabain–receptor complex. ATP binding to $(Na^+ + K^+)$-ATPase is generally studied in the presence of Mg^{2+}-chelating agents[37,49]. But studies on ouabain binding are performed in the presence of high concentrations of Mg^{2+}. It is possible therefore, that the effectiveness of K^+ and Na^+ as modifiers of ouabain binding was influenced by the Mg^{2+} concentration. Indeed, an effect of Mg^{2+}, which decreases the effectiveness of K^+ and Na^+ as inhibitors of [^{3}H]ouabain binding, can be demonstrated (Fig. 10).

Effects of Ca^{2+} on ouabain binding

Calcium even in low concentrations inhibits the hydrolytic activity of $(Na^+ + K^+)$-activated ATPase[5,30,49]. Calcium alone, or $Mg^{2+} + Ca^{2+}$, does not promote ouabain binding. Ca^{2+} does, however, substitute for Na^+ in ATP-dependent ouabain binding (Fig. 11): Ca^{2+} raises the affinity of the receptor for the cardiac steroid in the presence of $(Mg^{2+} + ATP)$.

If K^+ is present, the effect of Ca^{2+} is even more pronounced. 5 mM Ca^{2+} decrease the dissociation constant from $26 \cdot 10^{-8}$ M (in the presence of $Mg^{2+} +$ ATP + 5 mM K^+) to $2.8 \cdot 10^{-8}$ M. This finding is in agreement with pharmacological

studies showing a K^+–Ca^{2+} antagonism on the positive inotropic action of ouabain on heart muscle[3].

Binding of $^{45}Ca^{2+}$ to $(Na^+ + K^+)$-activated ATPase was studied, because an interaction of Na^+-transport with Ca^{2+} transport has been reported[50,51]. In our studies we found at least two different Ca^{2+}-binding sites, the high affinity site having a dissociation constant of $6.2 \cdot 10^{-4}$ M. Ouabain and any combination with Mg^{2+}, ATP and Na^+ did not influence the Ca^{2+} receptor site. It appears, that this Ca^{2+} binding site is not related to $(Na^+ + K^+)$-ATPase activity.

DISCUSSION

The studies reported in this paper show in agreement with previous work[14,18,28,34] the existence of one single type of ouabain receptor under special conditions, *i.e.* in the presence of $(Mg^{2+} + P_i)$ or $(Mg^{2+} + Na^+ + ATP)$. In the absence of these ligands almost no ouabain binding occurs. Mg^{2+} or Mn^{2+} increase the affinity of the ouabain receptor by about 50–60 fold (Table I). This increase of the affinity of the ouabain receptor towards the cardiac glycoside may occur because the ouabain receptor (R) exists in two different conformational states (eqn. 2):

$$R_\alpha \rightleftharpoons R_\beta \tag{2}$$

where R_α represents the part of the receptor with low affinity and R_β the part of the receptor with high affinity. It might be possible that Mg^{2+} and univalent cations affect the equilibrium between both states. If this model were valid, the changing affinities of the receptor for ouabain should be reflected in a curved line in the Scatchard plot under certain conditions (*i.e.* where a mixture of R_α and R_β exists); the total number of binding sites, however, should remain constant.

In agreement with his assumption, a curved line in the Scatchard plot[52] has been observed in the presence of 3 mM Mg^{2+} (Fig. 2). The curved line in the Scatchard plot disappeared and one single high affinity site appeared, when P_i was present in addition to Mg^{2+}. The total number of ouabain binding sites was not altered by P_i. This result may indicate that under the latter conditions all of the ouabain receptor sites are in the high affinity (R_β) conformational state.

The equilibrium between the low affinity state (R_α) and the high affinity state (R_β) is influenced by ligands and substrates of $(Na^+ + K^+)$-activated ATPase: The R_α-state is favoured by univalent cations such as K^+ and its congeners (Fig. 6), by Na^+ in the presence of Mg^{2+} and of $(Mg^{2+} + P_i)$ (Fig. 5), and by nucleotides which bind to the nucleotide binding site, but are not hydrolyzed, *i.e.* ADP–C–P, ATP–OCH_3 (Table I). It appears that all of the receptor is in the R_β state only under two conditions: in the presence of $(Mg^{2+} + P_i)$ and $(Mg^{2+} + Na^+ + ATP)$. Only under these special conditions the very low-value of 13 nM has been found (Table I). It has been demonstrated in previous work[53–55,59], that $(Na^+ + K^+)$-ATPase can be phosphorylated from P_i and ATP. When phosphorylation from nucleotidetriphosphates cannot occur, Na^+ does not stimulate ouabain binding but rather inhibits it (Table I). From this finding it is probable that a phosphorylated intermediate is involved in the Na^+-stimulated pathway of ouabain binding. This property was described by Tobin and Sen[22] as Na^+-activated and Na^+-inhibited pathway of ouabain binding.

When the ouabain receptor is more in the R_β state, K^+ is less effective in shifting the receptor towards the R_α state. This can be concluded from the fact that the change of K_D values by K^+ is more pronounced in the presence of Mg^{2+} alone than in the presence of $(Mg^{2+}+P_i)$ (Fig. 7). A change in the apparent affinity of Na^+ and K^+ for (Na^++K^+)-ATPase with different Mg^{2+} concentrations is demonstrated in Fig. 10 and has also been reported by Tobin and Sen[22] and Skou *et al.*[56].

From determinations of the association and dissociation rate constants of the ouabain–receptor complex it appears that the ouabain–receptor complex once formed, behaves identically irrespective of whether K^+ is present or not (Fig. 8). Differences, however, have been noted when the dissociation velocity was studied in the presence of EDTA[57].

The R_α and R_β conformational states of the ouabain-receptor are different from the E_1 and E_2 conformational states of the enzyme, although some similarities are evident: binding of nucleotides to the nucleotide receptor site, which favours the E_1 conformational state[58] reduces ouabain binding (R_α) (Table I). Na^+ which favours the E_1 state[37,47] also reduces the ouabain binding in the presence of Mg^{2+} (Fig. 5). Whereas Mg^{2+} which is assumed to induce the E_2 state increases ouabain binding (R_β). Mg^{2+} and Ca^{2+} reduce the affinity of ADP for the nucleotide receptor site[44,47], probably due to a shift of the enzyme into the E_2 conformational state. In agreement therewith Ca^{2+} increases the affinity for ouabain[49,14,24,29] (Fig. 11). However, the effects of K^+ and its congeners on ouabain binding cannot be classified within the E_1–E_2 scheme, because according to this scheme K^+ should increase ouabain binding. Apparently, this is not the case (Figs 6–8). It seems possible, however, that K^+ induces a conformational state of (Na^++K^+)-ATPase different from the E_1 and E_2 states. Hints for this assumption arise from the finding that Na^+ counteracts the effects of K^+ on ATP and ADP binding[37,47] but only partially abolishes the action of Mg^{2+} on ADP binding[47].

ACKNOWLEDGEMENT

The support of this work by the Stiftung Volkswagenwerk by a SORVALL RC-2B refrigerated centrifuge is gratefully acknowledged.

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