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

III. ON THE STABILITY OF THE OUABAIN RECEPTOR AGAINST PHYSICAL TREATMENT, HYDROLASES AND SH REAGENTS*

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SUMMARY

The stability of partial reactions of $(Na^+ + K^+)$-ATPase (nucleotide binding, phosphorylation, ouabain binding and potassium phosphatase) against the inactivation of $(Na^+ + K^+)$-ATPase by 5,5′-dithio-bis-(2-nitrobenzoic acid), ethacrynic acid, phospholipase A and trypsin were investigated. The properties of the ouabain receptor (affinity for the glycoside and binding capacity) were found to be relatively stable after pretreatment with the inactivating substances. The sensitivity of potassium phosphatase against pretreatment by SH reagents and hydrolytic enzymes was comparable to that of the $(Na^+ + K^+)$-ATPase activity. These findings confirm the concept that $(Na^+ + K^+)$-ATPase may be composed of subunits.

INTRODUCTION

Active transport of Na^+ and K^+ through cellular membranes is catalyzed by $(Na^+ + K^+)$-activated ATPase (E.C. 3.6.1.3)[1,2]. This enzyme is supposed to consist of subunits[3]. Some evidence for the correctness of this assumption may come from the work of Kyte[4] and Hokin *et al.*[5] who found in highly purified $(Na^+ + K^+)$-ATPase preparations two polypeptides. However, it could not be clarified, whether both polypeptides are part of $(Na^+ + K^+)$-ATPase. In a recent paper Shamoo and Albers[6] report the isolation of a Na^+ selective ionophoric material from $(Na^+ + K^+)$-ATPase by tryptic digestion. This paper may provide an additional hint for a possible subunit structure of the $(Na^+ + K^+)$-ATPase.

If the concept of the subunit structure of $(Na^+ + K^+)$-ATPase were correct, the subunits could have different stabilities against various agents. Because of this, we studied the viability of the ouabain-receptor and of partial reactions of $(Na^+ + K^+)$-ATPase against physical alterations, SH reagents and some hydrolytic enzymes. Specific binding of ouabain has been found to be directly related to $(Na^+ + K^+)$-ATPase activity[7,8]. This paper reports that the ouabain binding capacity and the

Abbreviation: DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid).

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properties of the ouabain receptor remain relatively constant even if the activity of $(Na^+ + K^+)$-ATPase decreases considerably.

MATERIALS

[^{3}H]Ouabain with a specific activity of 13 Ci/mmole was obtained from New England Nuclear, Dreieichenhain, Germany. [^{14}C]ADP with a specific activity of 535 Ci/mole and [γ-^{32}P]ATP (spec. act. 300–5000 Ci/mole) was from Amersham-Buchler, Braunschweig, Germany.

Triton X-100 was a product of Koch-Light Laboratories Ltd, Colnbrook, England, ethacrynic acid was obtained from Merck Sharp and Dohme, Rahway, N.J., USA; DTNB (5,5'-dithio-bis-(-2-nitrobenzoic acid)) and phospholipase A from *Crotalus terr. terr.* (EC 3.1.1.4) was from Boehringer, Mannheim, Germany; trypsin (EC 3.4.4.) from E. Merck, AG., Darmstadt, Germany. Trypsin inhibitor from hen egg white was from Boehringer, Mannheim.

All other chemicals were obtained through Boehringer, Mannheim and E. Merck AG., Darmstadt.

METHODS

Preparation and quantitation of enzyme

$(Na^+ + K^+)$-activated ATPase from beef brain was prepared as described previously[9]. $(Na^+ + K^+)$-ATPase activity was measured with the coupled optical assay[9]. The reaction was continously recorded and corrected for a Mg^{2+}-activated ATPase by inhibition of $(Na^+ + K^+)$-ATPase with 10^{-4} M ouabain. One enzyme unit is defined as the amount of enzyme hydrolysing 1 μmole ATP per min at 37 °C. Protein was quantitated by the procedure of Lowry *et al.*[10].

Assay of ouabain binding by a rapid centrifugation method is described in great detail in previous papers in this journal[8,26].

In brief: 0.2–0.4 mg enzyme protein were incubated unless otherwise indicated in 50 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–PO_4 and varying amounts of [^{3}H]ouabain at 37 °C until equilibrium was reached. The reaction was stopped by freezing in liquid air and rapid centrifugation at $80000 \times g$ for 30 min. Radioactivity in the sediment was counted after dissolving, in a liquid scintillation counter.

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 unlabelled ouabain.

Assay of [^{14}C]ADP binding to $(Na^+ + K^+)$-ATPase was measured essentially in the same way as [^{3}H]ouabain binding. In detail it is described in a previous paper[11]. The incubation was carried out at 0 °C for 0.5–3 min with 1–2 mg enzyme protein that had been washed twice with 10 mM Tris–EDTA and twice with distilled water. Results were corrected for unspecific [^{14}C]ADP binding by subtracting the value obtained in the presence of 10 mM unlabelled ADP.

The preparation of [^{32}P]phosphoprotein is described in detail elsewhere[8]. Unless otherwise indicated, the incubation medium contained 100 mM imidazole–HCl, pH 7.25, 5 mM $MgCl_2$, 1 μM [^{32}P]ATP and 100 mM NaCl or 10 mM KCl, respectively,

and about 1 mg of enzyme protein, 0 °C for 30–60 s. The reaction was stopped by addition of 5 ml cold trichloracetic acid (5% w/v) which contained unlabelled ATP and P_i; the solution was then centrifuged at $200 \times g$. The values in the presence of 10 mM KCl were subtracted from those in the presence of 100 mM Na^+. Previous experiments had shown that the high Mg^{2+} concentrations slowed down the rate of phosphorylation, but not the maximal number of phosphorylation sites. So it became possible to compare the rate of phosphorylation.

K^+-dependent phosphatase activity was measured by incubating 0.03–0.06 mg enzyme protein for 10 min in 100 mM Tris–HCl pH 7.8, 3 mM $MgCl_2$, 10 mM KCl, 5 mM Tris-*p*-nitrophenylphosphate in a total volume of 1 ml at 37 °C. The reaction was stopped by addition of 1 ml 1 M NaOH. The activity in the presence of 10^{-4} M ouabain was subtracted. Activity was calculated from the absorption at 405 nm.

RESULTS

(1) Properties of the non-treated ($Na^+ + K^+$)-ATPase from beef brain (control)

Nucleotide binding capacity. Nucleotide binding capacity of ($Na^+ + K^+$)-ATPase at 0 °C was repeatedly found to be 106 pmoles per enzyme unit. Binding capacity was calculated from the intercept in the Scatchard-plot[12] with the ordinate. [^{14}C]ADP was used as substrate for this determination, because ADP is a cosubstrate of the Na^+-dependent ATP–ADP transphosphorylation reaction, a part of the overall reaction[13]. Moreover it is a competitive inhibitor of ($Na^+ + K^+$)-ATPase[14]. The dissociation constant of the ADP–enzyme complex amounted to $0.6 \cdot 10^{-6}$M.

Amount of [^{32}P]phospho-intermediate. At 0 °C the [^{32}P]phospho-intermediate amounted to 80–90 pmoles per enzyme unit of ox brain cell membranes.

K^+-phosphatase activity. A K^+-activated phosphatase is believed to represent the terminal step of ($Na^+ + K^+$)-activated ATPase. With *p*-nitrophenylphosphate as substrate, phosphatase activity at 37 °C was between 0.05–0.4 I.U. per unit ($Na^+ + K^+$)-ATPase.

Ouabain-binding capacity. Ouabain-binding capacity of beef brain microsomes was measured to be 130 pmoles per unit ($Na^+ + K^+$)-ATPase. This binding capacity agrees with that reported by Hansen[7]. The dissociation constant in the presence of ($Mg^{2+} + P_i$) was $1.25 \cdot 10^{-8}$ M.

(2) Effects of physical treatment on partial reactions

Heat denatured microsomes lose ($Na^+ + K^+$)-ATPase activity parallel with the ouabain binding capacity. The same is true for repeated freezing and thawing: After 4 times freezing at −15 °C in 0.01 M imidazole–HCl, pH 6.5, and thawing, ($Na^+ + K^+$)-ATPase had lost 31% of its activity and the ouabain binding capacity had decreased by 26%.

(3) Treatment with phospholipase A

Incubation of beef brain microsomes with phospholipase A from *Crotalus terr. terr.*, an enzyme which cleaves the ester linkage at C_2 of phosphatides and forms lysophosphatides, resulted in a time dependent inactivation of ($Na^+ + K^+$)-ATPase and K^+-phosphatase activities. The amount of maximal nucleotide binding sites, ouabain binding sites and ^{32}P-labelled intermediate remained (if related to the protein

content) constant and so did the dissociation constants (K_D) (Table I). Phospholipase treatment apparently interrupts the overall reaction after the phosphorylation reaction.

TABLE I

EFFECT OF PHOSPHOLIPASE A ON $(Na^+ + K^+)$-ATPase AND ITS PARTIAL REACTIONS

30 ml enzyme suspension ($(Na^+ + K^+)$-ATPase activity 1.86 units/mg protein) were incubated for 30 min in 30 mM imidazole–HCl, pH 7.25, 3 mM $CaCl_2$ and 50 units phospholipase A at 37 °C. The reaction was stopped by addition of 30 ml 0.1 M Tris–EDTA, 0 °C. Then the protein was washed twice with distilled water and finally suspended in 0.01 M imidazole–HCl, pH 6.5. Further details are given in Methods.

	Control	*Phospholipase A treated protein*
$(Na^+ + K^+)$-ATPase activity (units/mg protein)	1.86	0.66 (= 36%)
K^+-phosphatase (units/mg protein)	0.84	0.26 (= 35%)
ADP-binding sites (pmoles/mg protein)	197	177 (= 90%)
ADP-binding sites (pmoles/unit)	106	262
Dissociation constant (K_D ADP $\times 10^{-6}$ M)	0.56	0.5
^{32}P-labelled intermediate formation (pmoles/mg protein)	148.2	136.4 (= 92%)
^{32}P-labelled intermediate formation (pmoles/unit)	79.7	206.8
Ouabain-binding sites (pmoles/mg protein)	242	242
Ouabain-binding sites (pmoles/unit)	131	361
Dissociation constant (K_D ouabain $\times 10^{-8}$ M)	1.4	1.32

(4) Treatment with trypsin

Treatment of the $(Na^+ + K^+)$-ATPase with trypsin resulted in rapid inactivation of enzymatic activity (Fig. 1)[15]. After addition of trypsin inhibitor (0.5 mg = 4000 units) there was no further inactivation. The trypsin-treated protein was washed in 0.01 M imidazole–HCl, pH 6.5, three times to remove any soluble protein. Although

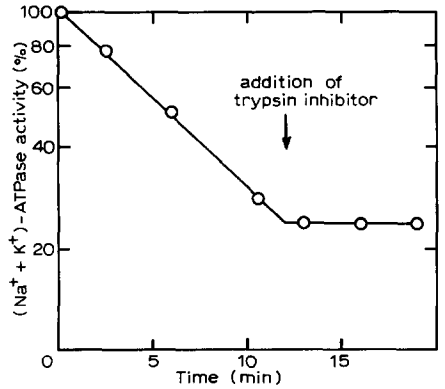


Fig. 1. Time dependent inactivation of $(Na^+ + K^+)$-ATPase by trypsin. 80 mg enzyme protein (ox brain, $(Na^+ + K^+)$-activated ATPase activity = 3.9 units/mg protein) were incubated in 25 mM Tris–HCl, pH 8.0 and 900 units/mg protein of bovine pancreas trypsin at 22 °C for the indicated time. After 12 min 0.5 mg of trypsin inhibitor was added (8000 units/mg protein).

the trypsin treatment had decreased $(Na^+ + K^+)$-ATPase and K^+-phosphatase activity to 29% and 21%, respectively, the maximum number of ouabain binding sites remained constant (Table III). The binding sites for [^{14}C]ADP had decreased slightly to 72% of control. The dissociation constant of ADP and ouabain binding were the same as in the control. The ^{32}P incorporation from [^{32}P]ATP was not changed by the trypsin treatment either. Trypsin treatment apparently interrupts the overall reaction at a relatively late step, possibly the dephosphorylation step.

(5) *Treatment with ethacrynic acid*

It has been reported that ethacrynic acid inhibits the phosphorylation parallel with the ATP hydrolysing activity of $(Na^+ + K^+)$-ATPase (Banerjee *et al.*[16]). If this were true, the binding sites for ouabain should decrease as well (assuming that the cardiac glycoside binds to the phosphorylated intermediate only). In order to test this, we inactivated the enzyme protein in the presence of 15 mM ethacrynic acid for 24 min at 37 °C. This treatment produced an inhibition of 43% of $(Na^+ + K^+)$-ATPase activity (Table II).

TABLE II

EFFECT OF ETHACRYNIC ACID ON OX BRAIN CELL MEMBRANES

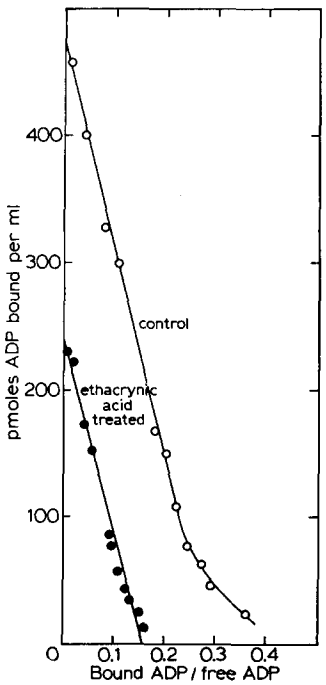
105 mg protein ($(Na^+ + K^+)$-ATPase activity= 3.0 units/mg protein) were incubated for 24 min at 37 °C in 25 mM imidazole–HCl, pH 7.25, 0.3 mM $MgCl_2$, 0.01 mM Tris–PO_4 and 15 mM ethacrynic acid. After this the treated and the control enzyme were washed twice in 10 mM Tris–EDTA and twice in distilled water at 80000 × *g* at 0 °C. The final protein was taken up in 0.01 M imidazole–HCl, pH 6.5.

	Control	*Ethacrynic acid treated protein*
$(Na^+ + K^+)$-ATPase activity (units/mg protein)	3.7	2.1 (= 57%)
K^+-phosphatase activity (units/mg protein)	0.2	0.14 (= 70%)
ADP-binding sites (pmoles/mg protein)	400	240 (= 60%)
ADP-binding sites (pmoles/unit)	108	114
Dissociation constant (K_D ADP × 10^{-6} M)	0.81	0.80
^{32}P-labelled intermediate formation (pmoles/mg protein)	380	330 (= 87%)
^{32}P-labelled intermediate formation (pmoles/unit)	103	157
Ouabain-binding sites (pmoles/mg protein)	500	460 (= 92%)
Ouabain-binding sites (pmoles/unit)	135	219
Dissociation constant (K_D ouabain × 10^{-8} M)	1.15	1.10

The K^+-phosphatase was also inhibited (30%). The binding sites for ADP (if related to the protein concent) were reduced in the same way as the overall reaction. There were 108 pmoles per enzyme unit in the control and 114 pmoles per enzyme unit in the treated protein. The dissociation constant remained unchanged (Fig. 2). It was quite surprising to notice that the ^{32}P-labelled intermediate formation was not reduced parallel with the $(Na^+ + K^+)$-ATPase activity as described by Banerjee *et al.*[16]. The reason for this apparent discrepancy was found out to be the short reaction time of 10 s, which was used by this group. As is evident from Fig. 3 phosphorylation still continues in ethacrynic acid treated samples, whereas in the control

samples [^{32}P]phosphate incorporation has already stopped. Treatment with ethacrynic acid apparently slows down the phosphorylation process. This finding may explain the controversial results obtained by Banerjee *et al.*[16] and us.

The maximal number of binding sites of ouabain was 92% of the control enzyme. The dissociation constant of the ouabain receptor remained unchanged (Table II).



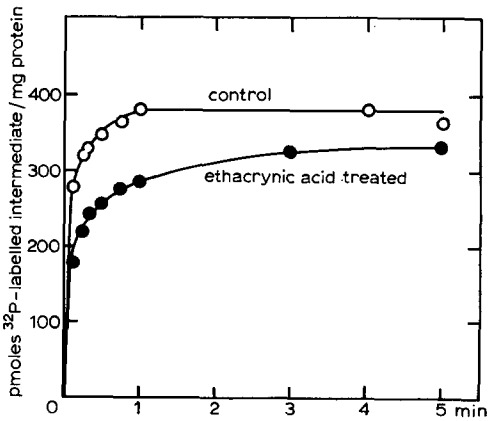


Fig. 2. ADP-binding to $(Na^+ + K^+)$-activated ATPase preparations. 1.17 mg protein (ox brain, $(Na^+ + K^+)$-ATPase activity= 3.7 units/mg protein) was incubated at 0 °C for 1 min in 50 mM imidazole-HCl, pH 7.5, and varying amounts of [^{14}C]ADP. The experiments with ethacrynic acid treated protein were performed with 1.0 mg protein ($(Na^+ + K^+)$-ATPase activity= 2.1 units/mg protein). See Table II. ADP binding to ethacrynic acid treated protein, ●—●; control, ○—○.

Fig. 3. [^{32}P]intermediate formation from [^{32}P]ATP. 0.5 mg of enzyme (ox brain, $(Na^+ + K^+)$-ATPase activity= 3.7 and 2.1 units/mg protein, respectively) were incubated at 0 °C in 100 mM imidazole-HCl, pH 7.25, 0.5 mM $MgCl_2$, 100 mM NaCl or 10 mM KCl, 10^{-5} M [^{32}P]ATP. Total volume 1.0 ml. The values in the presence of KCl were substracted from those in the presence of NaCl as unspecific ^{32}P binding.

(6) *Treatment with Ellman's Reagent*

Ellman's reagent, DTNB, a sulfhydryl inhibitor as ethacrynic acid[17], was used to study if the effects produced by ethacrynic acid depend on the nature of the sulfhydryl reagent. In general DTNB has similar effects on $(Na^+ + K^+)$-ATPase activity and its partial reactions, indeed (Table III): Again the ouabain binding sites were not impaired until most of the $(Na^+ + K^+)$-ATPase activity was lost. This happened after 24 min of incubation in 10^{-3} M DTNB at 37 °C. The ouabain receptor seems to be the most stable part of $(Na^+ + K^+)$-ATPase.

TABLE III

EFFECT OF PHOSPHOLIPASE A, ETHACRYNIC ACID, DTNB AND TRYPSIN ON OX BRAIN CELL MEMBRANES CONTAINING $(Na^+ + K^+)$-ATPase

Enzyme protein was incubated for 10–60 min with the substances listed below. The inactivation was stopped by repeatedly washing the protein at 80000 $\times g$ in aqua dest. Activities and binding sites left after treatment were compared as % of control.

	$(Na^+ + K^+)$-ATPase activity (%)	*K^+-phosphatase activity* (%)	*^{32}P-labelled intermediate formation* (%)	*ADP-binding capacity* (%)	*Ouabain-binding capacity* (%)
Phospholipase A (50 units per 38 mg membrane protein)	36	35	92	90	100
Ethacrynic acid (15 mM)	53	65	75	38	94
Ethacrynic acid (15 mM)	57	70	87	60	92
DTNB (0.02 μmole per mg protein)*	52	52	73	40	99
DTNB (0.05 μmole per mg protein)**	14	—	39	20	78
Trypsin (900 units per 80 mg protein)***	15	17	78	—	90
Trypsin (900 units per 80 mg protein)†	29	21	100	72	100

* 27 min of preincubation at 37 °C, pH 7.25.
** 24 min of preincubation at 37 °C, pH 7.25.
*** 20 min of preincubation at 22 °C, pH 8.0.
† 12 min of preincubation at 22 °C, pH 8.0.

DISCUSSION

The experiments reported in this paper demonstrate that the ouabain receptor is quite stable against various agents and treatments. So, it seems possible that the ouabain receptor represents a subunit of $(Na^+ + K^+)$-ATPase. From the experiments with DTNB and ethacrynic acid (Tables II and III) it is evident, that cysteine is not involved in ouabain binding. Furthermore, hydrolysis of peptide bonds in the neighbourhood of lysine residues by trypsin (if such bonds should be split in the ouabain receptor) does not impair ouabain binding. Cysteine and lysine residues have been found in the small chain and the large chain polypeptides of $(Na^+ + K^+)$-ATPase[4]. It is also apparent, that hydrolysis of phospholipids to lysophosphatides by phospholipase A from *Crotalus terr. terr.* does not affect ouabain binding. This finding, however, is in contrast to a report of Goldman and Albers[18], which shows that ouabain binding decreases together with $(Na^+ + K^+)$-ATPase activity and the ability to form a phosphorylated intermediate. These authors used phospholipase A from *Naja naja*. The differences in the findings of Goldman and Albers[18] and of us lay in the tissue source of the phospholipase A. It has been reported that phospholipase A from various sources has different substrate specifities[19]. In addition, it should be noted, that Goldman and Albers[18] studied ouabain binding in the presence of $(Mg^{2+} + Na^+ + ATP)$ whereas in this report, the Na^+ independent pathway of ouabain binding was used.

Although in untreated membrane preparations a linear relationship between ouabain binding capacity and nucleotide binding capacity can be observed[7,8,20,21], no such relationship exists any more in membrane preparations treated with SH-reagents (Table III). In agreement with previous work[1,2,22] we conclude from this that ouabain and nucleotide binding sites are separate from each other. Also the amount of phosphointermediate and ouabain binding capacity cannot be correlated, since a tremendous fall in phosphointermediate in a DTNB pretreated enzyme preparation did not affect ouabain binding capacity (Table III). This is in accordance with the findings of others[23,24]. Comparison of the nucleotide binding capacities and the ability for [^{32}P]phosphate incorporation after treatment of $(Na^+ + K^+)$-ATPase with SH-reagents shows that the amount of [^{32}P]phosphointermediate is about twice as high as the ADP binding capacity (Tables II and III). This finding could indicate that $(Na^+ + K^+)$-ATPase has more phosphate acceptor sites than nucleotide binding sites. This supposition would agree with the hypothesis of Robinson[25], which postulates a migration of phosphate from a glutamyl to a seryl residue of the enzyme during $(Na^+ + K^+)$-activated ATP hydrolysis.

In summary, the data in this paper show that ouabain binding capacity, nucleotide binding capacity and the capacity of phosphate acceptor can be altered by chemicals and enzymes independent of each other (Table III). This finding suggests a subunit structure of $(Na^+ + K^+)$-ATPase.

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