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TWO APPARENTLY DIFFERENT OUABAIN BINDING SITES OF (Na^+–K^+)-ATPase

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SUMMARY

1. The Scatchard plots of ouabain binding to (Na^+–K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) gave two straight lines. The dissociation constant and the binding capacity of the site of higher affinity were approx. 0.18 μM and 119 pmoles/mg protein, respectively, and those of the site of much lower affinity were 20 μM and 104 pmoles/mg protein, respectively.

2. Ouabain which was bound to the high-affinity site caused a marked reduction of both (Na^+–K^+)-ATPase activities in the presence of higher and much lower concentrations of ATP, respectively.

INTRODUCTION

Czerwinski *et al.*[1] and Neufeld and Levy[2,3] reported that two different independent enzymic sites, both of which are sensitive to ouabain, catalyze the hydrolysis of ATP in rat erythrocyte ghosts[1] and in calf brain microsomes[2,3]. Neufeld and Levy[3] suggested that the second ATPase may be related to a Na^+ pump such as that described by Hoffman and Kregnow[4]. Kanazawa *et al.*[5] and Hegyvary and Post[6] concluded both enzymic sites to be present in a single enzyme. But direct evidence showing whether both sites are related to the (Na^+–K^+)-ATPase reaction or whether the two sites are independent has not yet been obtained.

In this report the authors suggest, on the basis of ouabain binding experiments, that both enzymic sites are related to the (Na^+–K^+)-ATPase reaction.

METHODS

The method of partial purification of (Na^+–K^+)-ATPase from ox brain microsomes was reported previously[7]. (Na^+–K^+)-ATPase activity was approx. 60–70 μmoles ADP/mg protein under the standard conditions (pH 7.4 at 37 °C) as described[8].

The reaction mixture for ouabain binding experiment contained 5 mM $MgCl_2$, 140 mM sodium acetate, 14 mM KCl, 4 mM ATP, 0.25 mM Tris–EDTA, 190 mM sucrose, 40 mM Tris–acetate, 0.5 mg/ml of the ATPase protein and various concentrations of [^{3}H]ouabain (1–100 μM) in a final volume of 0.05 ml, at pH 6.1 and 37 °C. The binding reaction of [^{3}H]ouabain to the enzyme was started by the addition of

[^{3}H]ouabain; after 2 h of incubation, 3 ml of ice-cold unlabelled ouabain (pH 6.1 with 40 mM Tris–acetate) at a final concentration of 5 mM was added to the reaction mixture as a carrier. The binding of ouabain was a slow process, but after approximately 2 h of incubation a plateau level of binding was obtained (K. Taniguchi and S. Iida, unpublished results). The enzyme suspensions were immediately filtered through a membrane filter as described previously, and counted[9]. We regarded this amount of ouabain binding as the maximum.

The dissociation constants and the binding capacities were determined by plotting the data of the maximum amount of ouabain binding according to the method of Scatchard[10].

(Na^+–K^+)-ATPase activities were measured as the (Na^+–K^+)-dependent, ouabain-sensitive liberation of ADP and were coupled to the oxidation of NADH as described previously[8]. The assay conditions for the determination of (Na^+–K^+)-ATPase activity were nearly the same as those of the ouabain binding experiments. Other additions to the reaction mixture were 1.5 mM phosphoenolpyruvate, 0.2 mM NADH, 0.05 mg/ml pyruvate kinase (EC 2.7.1.40) and 0.025 mg/ml lactate dehydrogenase (EC 1.1.1.27); the final pH was adjusted to 6.1 at 37 °C. In the experiment described below, NADH oxidation was linear with time and was directly proportional to the amount of ATPase added, as described previously[8].

^{3}H-labelled ouabain (11.7 Ci/mmole) was obtained from New England Nuclear Boston, Mass., U.S.A. Ouabain was obtained from Tokyo Kasei Co., Tokyo, Japan. Pyruvate kinase from rabbit muscle and lactate dehydrogenase from pig muscle were obtained from Boehringer Mannheim, Tokyo, Japan. The other reagents were of reagents grade.

RESULTS AND DISCUSSION

The data of maximum ouabain binding to the enzyme preparations fell on two straight lines in the Scatchard plots. A typical example is shown in Fig. 1. From these slopes, the dissociation constants for ouabain were calculated to be 0.18 μM and 20 μM. The number of ouabain binding sites was obtained from the intercepts of the plots on the abscissa, as approximately 119 and 223 pmoles/mg protein (both the values of the dissociation constants and of the binding capacities were means of two different experiments on the same enzyme preparation). Thus, the number of the sites of the higher affinity was 119 pmoles/mg protein and the number of the sites of much lower affinity was 104 pmoles/mg protein, the latter value being obtained by subtraction of the number of high-affinity sites from 223 pmoles/mg protein.

From the Lineweaver–Burk plots of the (Na^+–K^+)-ATPase activities of our ATPase preparation, it was found that the maximum rate of ATPase activity and the apparent Michaelis constant at pH 6.1 and 37 °C were 2.4 μmoles/mg protein per h and 0.9 μM, respectively, over the low concentration range of ATP; and 41 μmoles/mg protein per h and 120 μM, respectively, over the high concentration range of ATP. This experiment confirmed the existence of two apparently different kinds of catalytic sites reported by several authors[1–3,5].

We investigated which catalytic site was inhibited by the ouabain bound to the high-affinity site. Both types of (Na^+–K^+)-ATPase activity were measured in the presence of 0.18 μM ouabain, at which concentration ouabain should bind to one

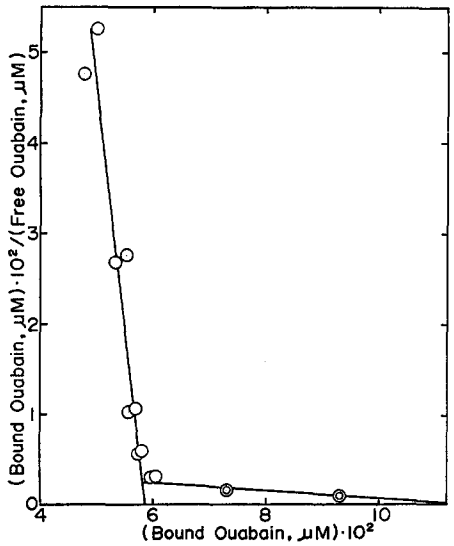

Fig. 1. Scatchard plots of ouabain binding to the (Na^+–K^+)-ATPase preparation from ox brain microsomes. Data were obtained by the millipore filtration method as described in the text. All experiments were performed in duplicate and the variations between duplicate samples were within ± 5%.

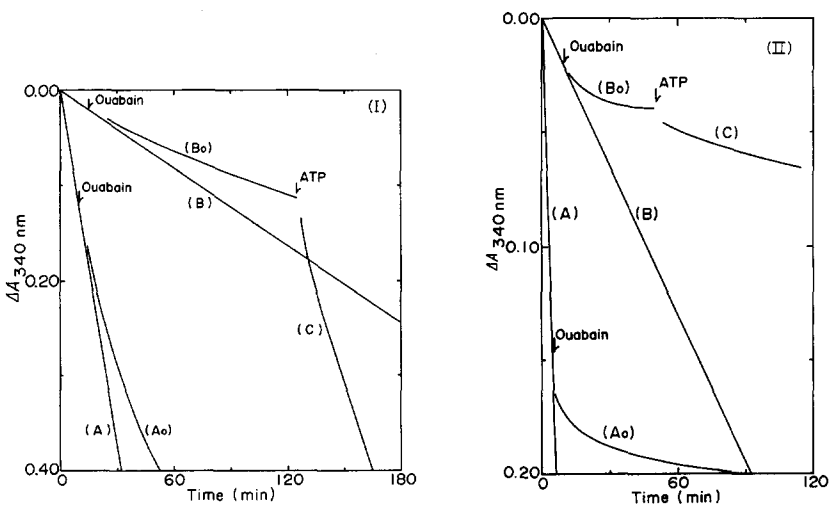

Fig. 2. (Na^+–K^+)-dependent ATPase activities of two apparently different catalytic sites and the effect of ouabain on those activities. The concentration of ATP was 1.8 μM for measuring the activity of one catalytic site with low K_m and low ATPase activity, and 745 μM for measuring the activity of the other catalytic site with high K_m and high ATPase activity. Additions of ouabain and further additions of ATP were made using 0.01-ml micropipettes, as indicated by the arrows. Other additions for the maintenance of constant ATP concentration during the assay and for measuring NADH oxidation are described in the text. The final volume of the reaction mixture was 1.56 ml at pH 6.1 and at 37 °C. (I) ATPase protein concentration; A, Ao, 3.1 μg protein/ml; B, Bo and C, 6.2 μg protein/ml. The concentration of ouabain was 0.18 μM. A, (Na^+–K^+)-ATPase activity was measured in the presence of 745 μM ATP; Ao, the ATPase activity was measured in the presence of 745 μM ATP and 0.18 μM ouabain. B, the ATPase activity was measured in the presence of 1.8 μM ATP; Bo, the ATPase activity was measured in the presence of 1.8 μM ATP and 0.18 μM ouabain. C, the ATPase activity was measured after the addition of 743.2 μM ATP after the ATPase activity in the presence of 1.8 μM ATP had been inhibited by 0.18 μM ouabain. (II) ATPase protein concentration was 9.5 μg protein/ml. The concentration of ouabain was 9.6 μM, if present, and other details were similiar to those in Fig. 2 I.

half of the high-affinity sites (the apparent dissociation constant for the high-affinity site was approx. 0.18 μM, as described above). A typical example was shown in Fig. 2 I. The specific activity was 32.3 ± 1.6 μmoles ADP/mg protein per h in the presence of 745 μM ATP (three observations from the same preparation). Additions of 0.18 μM ouabain caused a gradual decrease in the ATPase activity (Ao). It decreased to about 13.5 μmoles ADP/mg protein per h after 40 min incubation with ouabain. The specific activity of one catalytic site with a low apparent K_m value and low ATPase activity was 2.1 ± 0.10 μmoles ADP/mg protein per h (n = 3) in the presence of 1.8 μM ATP (B), and addition of 0.18 μM of ouabain also caused a gradual decrease in the activity (Bo). After 110 min incubation with 0.18 μM ouabain and 1.8 μM ATP, the specific activity was approximately constant at a level of 1.0 ± 0.1 μmole/mg protein per h (n = 3). Then the activity of the other site with a high K_m and high ATPase activity was measured by the addition of 743.2 μM ATP (C). The activity of this site was also inhibited. After 40 min incubation with 0.18 μM ouabain and 745 μM ATP, the specific activity was approximately constant at a level of 9.8 μmoles ADP/mg protein per h. This fact also suggests that the dissociation constant for the site with high affinity for ouabain is approx. 0.18 μM or less.

Similar experiments were performed in the presence of 9.6 μM of ouabain, at which concentration ouabain should bind mainly to the high-affinity site, as easily estimated from the plots (the apparent dissociation constant for the low-affinity site was approx. 20 μM as described above). The specific activity was approx. 32.3 μmoles ADP/mg protein per h in the presence of 745 μmoles of ATP, as described above, and addition of 9.6 μM ouabain caused a marked reduction in the ATPase activity as shown in Fig. 2 II (A, Ao). The specific activity of one catalytic site with a low apparent K_m value and low ATPase activity was approx. 2.1 μmoles/mg protein per h in the presence of 1.8 μM ATP (B), as described above, and addition of 9.6 μM ouabain also caused a marked reduction in activity (Bo). Then the activity of the other site with a high K_m and high ATPase activity was measured by the addition of 743.2 μM ATP (C). The activity of this site was also strongly inhibited.

These results strongly suggest that both catalytic sites are related to the (Na^+–K^+)-ATPase reaction rather than that the two sites are independent of each other, as suggested by Neufeld and Levy[2,3].

Two kinds of ouabain binding sites were clearly demonstrated in this experiment. The number of high affinity sites was approximately the same as that of the low affinity sites. In the absence of K^+, the number of low-affinity sites decreased to approximately one half (K. Taniguchi and S. Iida, unpublished results). Koshland[11] describes that the Scatchard plot is concave upward when there is a negative cooperativity (when the first molecule of ligand bound makes it more difficult for the next to bind) or when there is heterogeneity of binding sites. Our results may suggest that the existence of two apparently different ouabain binding sites is a reflection of a negative cooperativity, but we cannot exclude the possibility of a possible heterogeneity of the binding sites.

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