OUABAIN-SENSITIVE ^{42}K BINDING TO Na^+,K^+-ATPase PURIFIED FROM CANINE KIDNEY OUTER MEDULLA

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SUMMARY

Specific and ouabain-sensitive potassium binding to Na^+,K^+-ATPase was directly observed by centrifugation method with the purified enzyme and ^{42}K. The specific binding reached to saturation level at concentrations more than 0.2 mM KCl and the level was 6.2 nmol per mg ATPase with specific activity of 1470 μmol Pi/h·mg. The binding level, however, was proportional to the enzyme unit used. Simultaneous determination of ^{42}K binding and [^3H]ouabain binding showed that two mol of potassium binding were blocked by one mol of ouabain binding per 3.2×10^5 g enzyme. Although the apparent dissociation constant of the specific potassium binding was estimated at about 50 μM, Scatchard plot of the binding revealed non-linear relationship suggesting that the two potassium sites existed on one catalytic unit of enzyme would be not equivalent but cooperative.

The interaction of Na^+ and K^+ to Na^+,K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) including the affinity change of the enzyme to the ions has been studied indirectly by measuring the effects of Na^+ and K^+ on the partial reactions and the enzyme inactivations by inhibitors (1,2). In spite of necessity for the direct determination of cation binding to the enzyme, there has been only a few reports on this kind of investigation (3-6) because of the difficulty that the low level of specific cation binding is hidden among the high level of experimental error of nonspecific binding by the use of low purity enzyme preparation for the not so much difference of affinities between the specific and nonspecific binding site. Recently, application of a purified Na^+,K^+-ATPase to the sodium binding was reported (7). The present communication describes the demonstration of an ouabain-sensitive ^{42}K binding to a purified Na^+,K^+-ATPase (8) as well as number of the site and the specific features of the potassium binding.

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MATERIALS AND METHODS

Enzyme: The Na^+,K^+-ATPase was purified from canine kidney outer medulla by deoxycholate(DOC)-treatment and sodium dodecyl sulfate(SDS)-treatment with zonal centrifugation. The purification methods and characteristics of the enzyme was described elsewhere (8). The specific activities of the Na^+,K^+-ATPase preparations ranged from 1200 to 1500 μmol Pi/h·mg and ouabain-insensitive activity was less than 0.3 % of the total ATPase.

Radioactive materials: ^{42}KCl obtained from Japan Atomic Energy Research Institute was diluted 10 times with cold one. The specific radioactivity was about 0.3 μCi/μmol at the start of counting. [^{3}H]Ouabain was obtained from New England Nuclear (Lot Number:747-136) and diluted to the specific radioactivity of one μCi/μmol. The radioactive purity was determined to be 92 % by the adsorption procedure with the excess of Na^+,K^+-ATPase, and taken into account in the calculation of the binding.

^{42}K Binding: The ouabain-sensitive ^{42}K binding was determined by the similar procedure to the [^{3}H]-labeled cardiac glycoside binding (9). One milligram of Na^+,K^+-ATPase was preincubated in a polycarbonate centrifuge tube in a total volume of 0.9 ml containing 50 μmol imidazole-glycylglycine buffer, pH 7.2 (at 25°), 1 μmol EDTA and various ligands when necessary, in the presence or absence of 0.1 mM ouabain at 37° for 10 min. After the tubes were rapidly cooled in an ice bath, 0.1 ml of 2 mM ^{42}KCl was added to the mixture and the incubation lasted at 0° for a few min. The binding reaction was terminated by centrifugation at 40 000 rpm in a pre-cooled Beckman rotor Type 40 at 0° for 10 min. After removal of the supernatants, the precipitates were dissolved in 0.3 ml of 2 % SDS by smashing with glass rods and heating for 2 min, and transferred to scintillation vials containing 10 ml of dioxane scintillation cocktail. The radioactivities were counted in Beckman liquid scintillation spectrometer and the counts were corrected for ^{42}K decay with elapsed time for 10 min interval.

[^{3}H]Ouabain binding: The measurement of the [^{3}H]ouabain binding followed the method of Matsui and Schwartz (9) with slight modification and was performed simultaneously with the ^{42}K binding by using of [^{3}H]ouabain in place of cold one in the preincubation mixture. The radioactivity which was determined with 100 mM NaCl and no other ligand, was assumed to be nonspecific value mainly due to the unbound [^{3}H]ouabain in the water space of the precipitate. The specific binding was calculated by subtracting the nonspecific value from total binding.

RESULTS AND DISCUSSION

Detection of ouabain-sensitive ^{42}K binding ^{42}K binding under various ligand conditions were measured at a constant KCl concentration (0.2 mM) and compared with [^{3}H]ouabain binding determined simultaneously. As shown in Table I, 16.4 nmol ^{42}K binding per mg protein was observed under no ligand condition and the binding level was decreased to 8.1 nmol by the addition of 5 mM Mg^{2+}. In the presence of ouabain with the system containing Mg^{2+}, the level was further decreased to 4.0 nmol ^{42}K/mg. The difference of ^{42}K binding between the absence or presence of ouabain, i.e., an ouabain-sensitive ^{42}K

Table I Effect of Various Ligands on ^{42}K Binding to Na^+,K^+-ATPase in the Absence or Presence of [^{3}H]Ouabain

Measurement of the binding of ^{42}K and [^{3}H]ouabain was performed as described under Materials and Methods using the DOC-treated enzyme with specific activity of 1280 μmol Pi/h·mg. ATP and Pi were Tris-salts. The individual values are averages of duplicate experiments and the deviations of values were less than 3 %.

Addition	^{42}K Binding (nmol/mg protein)			[^{3}H]Ouabain binding (nmol/mg protein)	
	-Ouabain	+Ouabain	Ouabain-sensitive	Total	Specific
None	16.4	12.8	3.6	4.6	2.5
10 mM KCl	7.5*	7.4*	0.1	2.2	0.1
100 mM NaCl	4.2	4.2	0	2.1	0
0.2 mM Pi	12.2	11.4	0.8	2.7	0.6
0.2 mM ATP	13.9	11.9	2.0	4.1	2.0
5 mM $MgCl_2$	8.1	4.0	4.1	4.6	2.5
" + 10 mM KCl	4.2	4.2	0	3.2	1.1
" + 100 mM NaCl	3.9	3.9	0	2.4	0.3
" + 0.2 mM Pi	5.6	4.3	1.3	4.7	2.6
" + 0.2 mM ATP	6.0	4.2	1.8	4.8	2.7

* These values were calculated by the original specific radioactivity of ^{42}K without taking account of the dilution. The difference (3.3 nmol) of values between the addition of 10 mM KCl or 100 mM NaCl may reflect the increase of nonspecific ^{42}K binding at the high concentration of KCl and, therefore, the nonspecific binding at 10 mM KCl is calculated to be 165 nmol/mg with taking account of dilution factor of 50.

binding (4.1 nmol/mg) was 1.7 times as much as the specific [^{3}H]ouabain binding (2.5 nmol/mg). Addition of 100 mM Na^+ to the system containing 5 mM Mg^{2+} decreased also the ^{42}K binding to 3.9 nmol/mg which was almost the same level as observed with Mg^{2+} plus ouabain. The ratio of radioactivity found in the pellet per mg enzyme to that in the supernatant per ml was about 2 % both in ^{42}K binding with Mg^{2+} plus ouabain system and in [^{3}H]ouabain binding with 100 mM NaCl system where the specific ouabain binding was completely inhibited (Table I). Although the percentage varied by enzyme preparations or centrifugal force (g × min) from 1.2 to 2.5 % (Table I,II and ref.7,9),

Table II Effect of Potassium Concentration on ^{42}K Binding to Na^+,K^+-ATPase in the Absence or Presence of [^{3}H]Ouabain

Conditions were the same as described under Materials and Methods except for the use of SDS-treated enzyme with specific activity of 1470 μmol Pi/h·mg and concentrations of ^{42}KCl indicated. Nonspecific level of [^{3}H]ouabain binding, 1.6 nmol/mg protein determined in the presence of 100 mM NaCl without $MgCl_2$, was subtracted from total binding for calculation of specific ouabain binding.

^{42}KCl (mM)	^{42}K binding (nmol/mg protein)			[^{3}H]Ouabain binding (nmol/mg protein)	
	-Ouabain	+Ouabain	Ouabain-sensitive	Total	Specific
0.02	1.7	0.3	1.4	4.7	3.1
0.05	4.0	0.8	3.2	4.7	3.1
0.1	6.3	1.5	4.8	4.6	3.0
0.2	9.0	3.0	6.0	4.6	3.0
0.5	14.0	7.8	6.2	4.7	3.1

these values were less than 3 % which was the value obtained in a control experiment with [^{3}H]water. Consequently, it is evident that the value of ^{42}K binding with Mg^{2+} plus ouabain system is mainly due to unbound potassium contained in the water space of the precipitate. With no ligand system, the ouabain-sensitive ^{42}K binding was also demonstrated to be 3.6 nmol/mg by the addition of 0.1 mM ouabain, and at the same time the specific binding of [^{3}H]ouabain was observed at saturation level (2.5 nmol/mg) (see Table I). Therefore, 5 mM Mg^{2+} did not affect the ouabain-sensitive ^{42}K binding, while Mg^{2+} displaced ouabain-insensitive ^{42}K binding almost completely.

Ratio of Ouabain-sensitive ^{42}K binding to [^{3}H]ouabain binding To estimate the saturation level of potassium binding and its dissociation constant, the ^{42}K binding was measured at various concentrations of KCl. As shown in Table II and Fig. 1A, the ouabain-sensitive ^{42}K binding reached to a saturation level at concentrations more than 0.2 mM KCl, while the radioactive potassium recovered in the precipitate with Mg^{2+} plus ouabain system

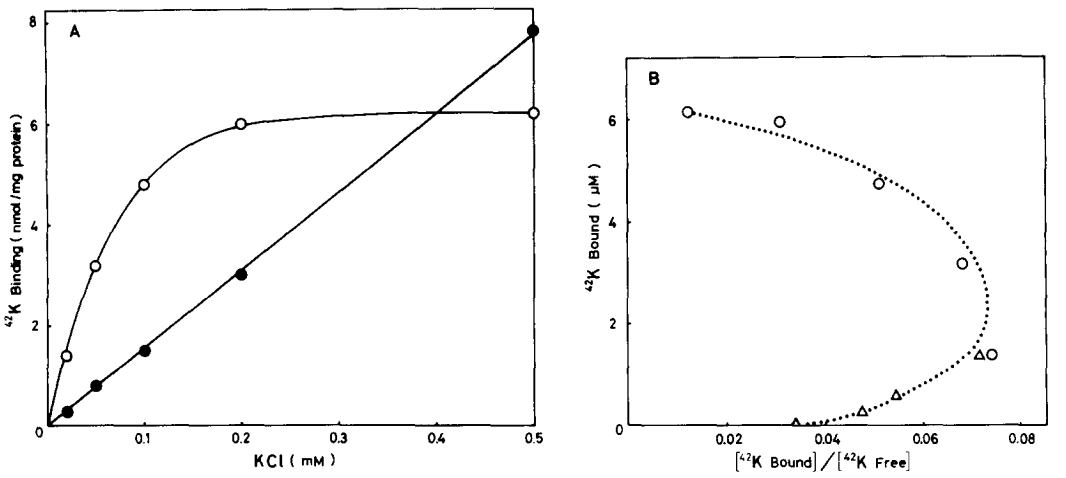

Fig. 1. A: Dependency of ouabain-sensitive ^{42}K binding on potassium concentration. From the data of Table II, the ouabain-sensitive ^{42}K binding (o), i.e., the difference between values in the absence and presence of ouabain, and the radioactive potassium in Mg^{2+} plus ouabain system (●) were plotted against potassium concentration. B: Scatchard plot of ouabain-sensitive potassium binding suggesting two binding sites with cooperative nature. The data for the ouabain-sensitive ^{42}K binding were plotted by Scatchard method; o, the same data from Table II; Δ, data from another experiment at lower potassium concentrations (2 to 20 μM). In order to adjust the difference levels of the total ^{42}K binding ($[M_B]$) between two experiments, a value of 0.41 nmol ^{42}K/mg at 20 μM KCl in the latter experiment was adjusted to 1.4 nmol/mg which was the value obtained at the same KCl concentration in the experiment of Table II. The dotted line indicates a theoretical curve calculated from $[M_B]$ obtained with Eq.4 where K_1 and K_2 were substituted with 100 and 20 μM respectively.

increased proportionally with increasing concentration of potassium in the reaction mixture suggesting the increase due to unbound ^{42}K in water space of the precipitate. The saturation binding level was dependent on the specific activity of Na^+,K^+-ATPase and about 4 pmol ^{42}K were bound per unit of the enzyme defined as μmol Pi liberated per h. The simultaneous determination of ^{42}K binding with [^{3}H]ouabain binding showed that two mol of potassium binding were blocked by one mol of ouabain binding per 3.2×10^5 g enzyme (Table II). An apparent dissociation constant (K_d) for the ouabain-sensitive binding was approximately 50 μM (Fig. 1A), but its complicated nature is discussed in the next section. On the other hand, the ouabain-insensitive ^{42}K binding was analyzed by Scatchard plot at higher concentration of KCl (0.02 to 20 mM) and

number of sites and K_d were estimated to be about 230 nmol/mg protein and 9 mM respectively (see footnote of Table I).

These results indicate the presence of two types of ^{42}K binding ; the one, ouabain-sensitive and relatively high affinity binding may represent a specific binding of potassium to Na^+,K^+-ATPase, and the other, ouabain-insensitive, Mg^{2+}-susceptible and lower affinity binding may represent nonspecific bindings of potassium probably to phospholipids as well as proteins. The data of the specific ^{42}K binding together with reports from various investigaters propose the following stoichiometrical relationship; the molar ratio of ^{42}K binding to ^{22}Na binding (7) and to ouabain binding (Table II ref. 7,9), phosphorylation (7,9,10), or ATP binding (10,11) is 2 : 3 : 1. This ratio agrees with the stoichiometry in the cation transport that 2 mol of K^+ and 3 mol of Na^+ are transported per one mol of ATP hydrolyzed (12).

Nature of ouabain-sensitive ^{42}K binding A Scatchard plot composed of the data for the ouabain-sensitive ^{42}K binding from Table II exhibited an unusual shape as seen in Fig. 1B. It is impossible to obtain number of the site and the K_d simply from this plot because the plot is not approximated with sum of a couple of straight lines. Analysis of the results, however, suggests that the two potassium sites existed on one catalytic unit of the ATPase are not equivalent but cooperative, e.g., binding of potassium to the first site increasing affinity for the second site. Assuming the following relationships :

$$E + M \rightleftharpoons EM \qquad \frac{[E][M]}{[EM]} = K_1 \qquad (1)$$

$$EM + M \rightleftharpoons EMM \qquad \frac{[EM][M]}{[EMM]} = K_2 \qquad (2)$$

$$[E_t] = [E] + [EM] + [EMM] \qquad (3)$$

where K_1 and K_2 denote K_d for the first and the second potassium binding respectively, [M] free potassium concentration, and $[E_t]$, [E], [EM], [EMM]

concentration of total, free, one potassium bound and two potassium bound enzyme respectively, we obtain total bound potassium $[M_B]$ as :

$$[M_B] = [EM] + 2[EMM] = \frac{[E_t]}{1 + K_1/[M] + [M]/K_2} + \frac{2[E_t]}{1 + K_2/[M] + K_1K_2/[M]^2} \quad (4)$$

Suppose $[M] \ll K_1, K_2$ or $[M] \gg K_1, K_2$, double reciprocal plots of $1/[M_B]$ vs. $1/[M]$ should show two straight lines and give K_1 or K_2 respectively from their slopes. Although estimation of the K_1 experimentally is rather difficult, the K_2 is roughly estimated at 10 - 20 μM from the data of Table II. By substituting K_1 and K_2 with 100 and 20 μM respectively, a curve calculated from $[M_B]$ obtained with Eq. 4 showed a reasonable simulation for the experimental values (Fig. 1B). Particularly, inversion of the theoretical curve, i.e., positive value for the slope of tangent, well explained the data from another experiment at low potassium concentrations less than 20 μM (Fig.1B).

The ouabain-sensitive potassium binding showed relatively broad pH dependency and the optimum pH (at 0^o) was about 7.2. The potassium binding was competitively decreased by the congeners of potassium, such as Tl^+, Rb^+, NH_4^+, Cs^+, Li^+ and Na^+. Although 5 mM Mg^{2+} was not affected the specific ^{42}K binding, 100 mM Mg^{2+} inhibited the potassium binding. The decrease of the potassium binding in the presence of ATP or Pi (Table I) is of particular interest because the decrease is due to the affinity change of the enzyme to potassium associated with its conformational change induced by ATP or Pi as substrate and modulator (9,10,13,14).

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