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PRELIMINARY COMMUNICATIONS

IDENTIFICATION WITH POTASSIUM AND VANADATE OF TWO CLASSES OF SPECIFIC OUABAIN BINDING SITES IN A (Na^+ + K^+)ATPase PREPARATION FROM THE GUINEA-PIG HEART

Théophile Godfraind, Alain De Pover and Di-Nzuzi Tona Lutete

Université Catholique de Louvain-Faculté de Médecine
Laboratoire de Pharmacodynamie Générale et de Pharmacologie
Avenue E. Mounier, 73-U.C.L. 7350 - B-1200 Bruxelles (Belgium)

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We have recently reported that the specific binding of [^{3}H]ouabain to human heart (Na^+ + K^+)ATPase occurred on high and low affinity binding sites. The dissociation constant (K_D) of [^{3}H]ouabain low affinity binding sites was close to ouabain K_i, an indication that these sites are involved in the inhibition of this enzyme by the glycoside. In guinea-pig heart microsomes, only one group of [^{3}H]ouabain binding sites were identified and the K_D of these sites was close to ouabain K_i (1). In intact guinea-pig heart, it was previously shown that [^{3}H]ouabain interacts with two groups of specific binding sites and that the proportion of the high affinity sites is increased by increasing extracellular K^+ (2,3).

We have now designed experiments in order to examine the binding of [^{3}H]ouabain to guinea-pig heart microsomes incubated with Mg^{2+}, ATP and Na^+ in the presence of K^+. In order to inhibit the hydrolysis of ATP resulting from the activation of (Na^+ + K^+)ATPase, vanadate -which also increases the affinity of (Na^++ K^+)ATPase for K^+ (4) -has been added to the incubation medium.

The results show the existence of [^{3}H]ouabain high affinity sites in guinea-pig heart microsomes treated by vanadate and potassium.

METHODS

Guinea-pig heart ($Na^+ + K^+$)ATPase has been prepared from NaI-and deoxycholate-treated homogenates (5). Enzyme assays were carried out as described previously,and the released inorganic phosphate was measured by the Fiske and Subbarow method (5). The specific activity was 0.5 enzyme unit/mg protein. (One ($Na^+ + K^+$)ATPase unit allows the release of 1 µmol inorganic phosphate per min). ($Na^+ + K^+$)stimulated ATP hydrolysis increased linearly with incubation time, at least up to 240 min.

[^{3}H]Ouabain binding was determined by a filtration technique described elsewhere (1). To prevent changes in ATP concentration in some experiments, creatine phosphate and creatine phosphokinase were added to the incubation medium. Reaction of creatine phosphate with the released ADP regenerates ATP (6). To prevent any effect of the released inorganic phosphate (P_i), excess of P_i was also added to the incubation medium.

Ammonium metavanadate (VO_3^-) was obtained from Merck and dissolved in 0.03 N NH_3 at a final concentration of 10^{-2}M.

RESULTS

The inhibition of guinea-pig heart ($Na^+ + K^+$)ATPase by various concentrations of VO_3^- has been studied. VO_3^- I_{50} was equal to 2 µM and the maximum inhibition was reached with 100 µM VO_3^-. In a first experiment, 0.01 µM [^{3}H]ouabain binding has been measured after 15 min incubation in a medium containing 3 mM Mg^{2+} and various VO_3^- concentrations. Half-maximal and maximal binding were observed at respectively 0.4 µM and 10 µM; higher concentrations of VO_3^- were less efficient.

VO_3^--supported [^{3}H]ouabain binding has been compared to the binding supported by other ligands with respect to the influence of Na^+ and K^+. As shown in Table I, Na^+ depressed the binding facilitated by both P_i and VO_3^- in the absence of ATP, whereas it enhanced the binding when ATP was present. On the other hand, K^+ inhibited in all the conditions so far examined.

The binding of [^{3}H]ouabain has been measured after an incubation of 2 hours in the presence of Mg^{2+}, Na^+, P_i, ATP and VO_3^- with or without K^+. The concentration of ATP was maintained at 3 mM with a regenerating system. The concentration of [^{3}H]ouabain was varied from 10^{-9}M to 10^{-5}M.

When the binding of [^{3}H]ouabain was measured in the absence of K^+,the Scatchard plot was linear indicating the existence of one class of binding sites with K_D equal to 0.13 µM (Fig. 1, left panel). When the binding of [^{3}H]ouabain was measured in the presence of 10 mM K^+ (Fig. 1, right panel), the Scatchard plot was upward-concave, suggesting the existence of two classes of binding sites. The maximum [^{3}H]ouabain binding capacity was not altered. There was a reduction of the affinity for

TABLE I : Influence of monovalent cations on specific [^{3}H]ouabain binding. The enzyme (0.08 mg/ml) was incubated for 15 min at 37°C in the presence of 1 mM EGTA, 20 mM Tris/maleate (pH 7.4), 0.01 µM [^{3}H]ouabain and, following the indications, 3 mM Mg^{2+}, 3 mM ATP, 3 mM P_i, 0.01 mM VO_3^-, with or without 100 mM Na^+ or 10 mM K^+.

	pmol [^{3}H]ouabain/enzyme unit (± S.E.M., n = 3)		
	without Na^+ and K^+	with Na^+ without K^+	with K^+ without Na^+
Mg^{2+}	0.09 ± 0.05	0.06 ± 0.03	0.12 ± 0.04
Mg^{2+} + P_i	7.43 ± 0.13	0.18 ± 0.07	0.67 ± 0.08
Mg^{2+} + VO_3^-	5.86 ± 0.03	0.37 ± 0.04	0.19 ± 0.05
Mg^{2+} + ATP	1.82 ± 0.10	7.50 ± 0.28	0.89 ± 0.04
Mg^{2+} + ATP + P_i	1.90 ± 0.04	8.64 ± 0.38	0.88 ± 0.09
Mg^{2+} + ATP + VO_3^-	2.47 ± 0.04	8.11 ± 0.16	0.27 ± 0.01

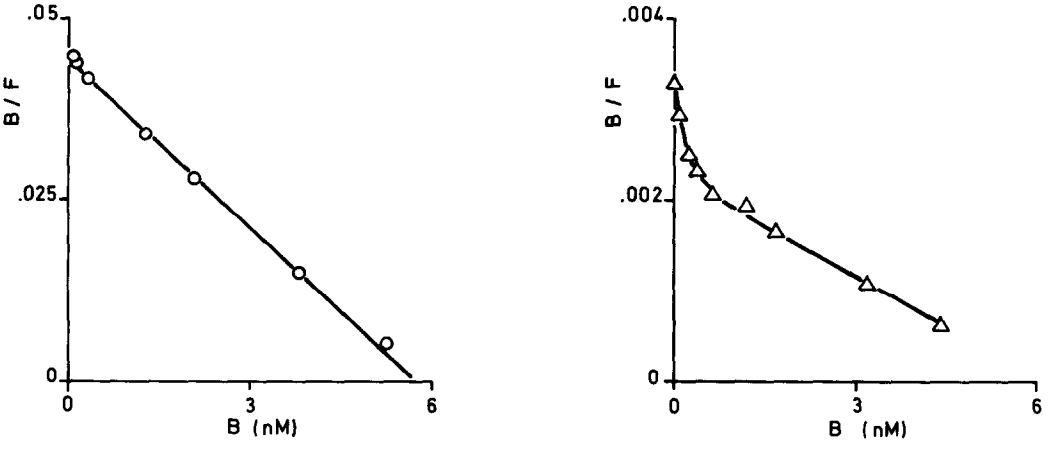

Fig. 1. : Scatchard plots for [^{3}H]ouabain binding. The enzyme (about 0.24 mg/3 ml) was incubated at 37°C for 2 h in the presence of 100 mM Na^+, 3 mM Mg^{2+}, 0.01 mM VO_3^-, 3 mM ATP, 5 mM P_i, [^{3}H]ouabain (0.019-19 Ci/mmol), 1 mM EGTA, 3 mM phosphocreatine, 50 µg creatine kinase (25 units/mg), 20 mM Tris/maleate (pH 7.4) and 10 mM K^+ (right panel) or without K^+ (left panel). The radioactivity bound in the presence of 10 mM K^+ was 221 cpm at 10^{-9}M (non-specific : 31 cpm) and 1105 cpm at 10^{-5}M (non-specific : 85 cpm). Each point is the mean of triplicate determination. S.E.M. did not exceed the diameter of the symbols.

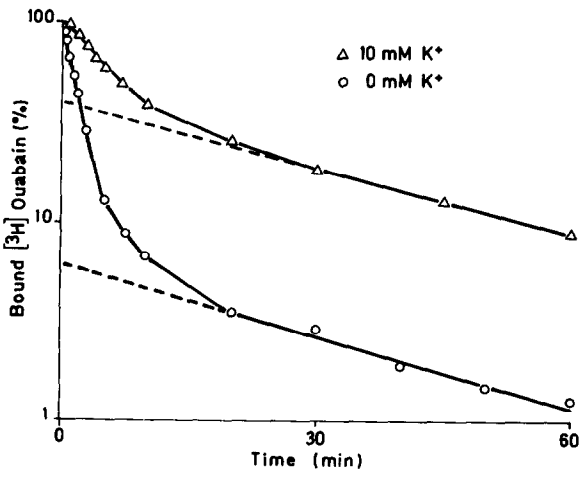

Fig. 2. : Time-course of [^{3}H]ouabain dissociation from (Na^+ + K^+)ATPase preparation. The enzyme (about 0.08 mg/ml) was incubated with 0.01 µM [^{3}H]ouabain (19 Ci/mmol) as described in the legend to fig. 1. After 2 h incubation, the specific binding was 24 pmol/mg protein without K^+ (6.920 cpm bound par ml filtered) and 2.2 pmol/mg with 10 mM K^+ (640 cpm bound/ml filtered). At this time, an excess of unlabelled ouabain was added and the amount of bound label was followed. The non-specific binding was 50 cpm per ml filtered. Each point is the mean from 3 experiments. S.E.M. did not exceed the diameter of the symbols.

[^{3}H]ouabain as shown by a 20-fold increase in the K_D for low affinity binding sites on which most of [^{3}H]ouabain binding occurred (K_D = 2.5 µM).

Observation of upward-concave Scatchard plot prompted experiments to study the rate of dissociation of the ouabain-enzyme complex formed in the absence and the presence of K^+. Therefore, 0.01 µM [^{3}H]ouabain was added to the incubation medium and 2 hours later, an excess of unlabelled ouabain was added to make the final concentration 0.2 mM. The amount of [^{3}H]ouabain remaining bound was estimated after various subsequent periods of time.

In the absence of K^+, the dissociation of [^{3}H]ouabain from guinea-pig heart microsomes occurred with a half-life of 1.5 min. There appears to be a slowly dissociating residual pool already noticed by Tobin et al (7) in guinea-pig heart (Na^+ + K^+)ATPase in the absence of vanadate.

In the presence of K^+, the dissociation of ouabain was markedly slower, the half-life being brought to 7 min. The proportion of the slowly dissociating pool increased from 6.3 to 40 %. This was mainly due to a depression by K^+ of the fast dissociating pool (from 11.3 pmole [^{3}H]ouabain/enz. unit at 0 K^+ down to 0.72 pmole [^{3}H] ouabain/enz. unit at 10 mM K^+) larger than that of the slowly dissociating one (from 0.67 pmole [^{3}H]ouabain/enz. unit at 0 K^+ down to 0.42 pmole [^{3}H]ouabain/enz. unit at 10 mM K^+). Furthermore, the presence of K^+ did not alter the half-life of the slow dissociation.

DISCUSSION

The results reported here show that guinea-pig heart (Na^+ + K^+)ATPase was slightly less sensitive to VO_3^- inhibition than lamb brain and dog kidney enzymes, I_{50} being 4-fold higher than that reported by others (4,9). They confirm that VO_3^- facilitates [^{3}H]ouabain binding in the presence of Mg^{2+} alone (10). The sensitivity to Na^+ exhibited by the [^{3}H]ouabain-enzyme binding formed with the VO_3^--enzyme has also been reported (11).

The presence of VO_3^- has not altered the apparent affinity of (Na^+ + K^+)ATPase for ouabain in the presence of Mg^{2+}, Na^+ and ATP. Indeed, in the absence of K^+, K_D estimate was close to the one already reported with an incubation medium without VO_3^- (1). The addition of VO_3^- has allowed to study the influence of K^+ on [^{3}H]ouabain binding in the presence of ATP and Na^+, as the K^+-stimulated ATP hydrolysis was prevented. In the presence of K^+, Scatchard plots and dissociation kinetics were modified indicating two classes of [^{3}H]ouabain binding sites which were not clearly observed in the absence of K^+.

Heterogeneity of [^{3}H]ouabain binding sites has been reported in human heart (1) beef brain (12) and rat brain (18). Potassium-induced change in heterogeneity has been reported by Hansen (12) and Choi and Akera (8). Our results show that [^{3}H]ouabain

high affinity and low affinity binding sites are distinguishable when the VO_3^--enzyme complex is incubated with K^+. K_D for the low affinity sites was equal to 2.5 µM, a value close to ouabain I_{50} estimated with 10 mM KCl in the absence of vanadate (unpublished observations). This indicates that the low affinity sites could be the inhibitory sites as it was already suggested in the human heart (1).

In intact isolated guinea-pig atria, two classes of [^{3}H]ouabain binding sites have been identified and potassium increased the proportion of the high affinity sites related to the stimulation of the ($Na^+ + K^+$) pump by ouabain (2,3). The results here reported indicate that a similar K^+ effect might occur on an isolated microsomal fraction of the guinea-pig heart.

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