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Effect of Monovalent Cations on the Ouabain Inhibition of the Sodium and Potassium Ion Activated Adenosine Triphosphatase*

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ABSTRACT: Inhibition of the (Na^+ + K^+)ATPase obtained from lamb brain by ouabain is first order in both enzyme and ouabain concentrations. This implies that the interaction of one ouabain per active site inhibits the enzyme. The second-order rate constant for inhibition, k_i, depends on both sodium and potassium ion concentrations. The rates of inhibition and binding are the same. The dependence of k_i on sodium and potassium ion concentrations is consistent with a mechanism in which ouabain interacts predominately with the phosphorylated enzyme with a rate constant k_i' which is independent of the monovalent cations bound. The rate constant for the binding of ouabain to the phosphorylated enzyme is 4.35×10^4 M^{-1} sec^{-1}. The dissociation constant, K_i, is less than 5×10^{-9} M.

The (Na^+ + K^+)ATPase (ATP phosphohydrolase, EC 3.6.1.3), which is believed to be an integral part of the alkali cation transport system of most mammalian tissues, is specifically inhibited by cardiac glycosides such as ouabain (Albers, 1967). There has been some controversy concerning the stability of the enzyme–ouabain complex. Several reports suggest binding is reversible (Glynn, 1964; Ahmed *et al.*, 1966; Hokin *et al.*, 1966; Tobin and Sen, 1970), difficult to reverse (Baker and Manil, 1968), or virtually irreversible (Albers *et al.*, 1968; Ellory and Keynes, 1969). It has been reported that potassium ion is at least partially competitive of ouabain binding and inhibition (Glynn, 1964; Ahmed *et al.*, 1966; Ellory and Keynes, 1969).

This communication hopes to help clarify some of the controversy over the stability of the enzyme–ouabain complex and the influence of sodium and potassium ions on the ATPase–ouabain interaction. A mechanism for the (Na^+ + K^+)ATPase is proposed based on the kinetics of ATP hydrolysis and the kinetics of ouabain inhibition and binding.

Experimental Section

Materials. Lamb brain (Na^+ + K^+)ATPase was prepared by the method of Nakao *et al.* (1965). The specific activity of several preparations was 2.0–2.7 μmoles of ATP hydrolyzed/mg of protein per min at 37°, and 95–98% of the ATPase activity was inhibited by ouabain. Protein was determined by the method of Lowry *et al.* (1951).

Tricyclohexylammonium phosphoenolpyruvate, disodium ATP, NADH, and rabbit muscle lactic dehydrogenase were obtained from Sigma Chemical Co. Ouabain was obtained from Aldrich Chemical Co. and [^3H]ouabain, 12 Ci/mmole, was purchased from New England Nuclear Corp. Rabbit muscle pyruvate kinase was prepared by the method of Tietz and Ochoa (1962). Tris-ATP was prepared by the method of Schwartz *et al.* (1962). Lactic dehydrogenase and pyruvate kinase were dialyzed against 0.01 M Tris, pH 7.0, to remove ammonium ion.

Assay of the (Na^+ + K^+)ATPase. In the usual assay procedure, the hydrolysis of ATP was coupled to the oxidation of NADH using pyruvate kinase and lactic dehydrogenase. The advantages of this assay procedure are that ATP hydrolysis can be monitored continuously and inhibitory buildup of ADP is prevented. Pyruvate kinase and lactic hydrogenase activities were always maintained in at least a 100-fold excess over ATPase activity. It was verified for all potassium ion concentrations used that a severalfold variation in the concentration of pyruvate kinase and lactic dehydrogenase had no effect on the ATPase kinetics. Oxidation of NADH was linear for over 2 hr and was directly proportional to the amount of ATPase added. Assay of the ATPase by orthophosphate production (Chen *et al.*, 1956) gave the same specific activity. Neither lactic dehydrogenase nor pyruvate kinase is affected by 10^{-4} M ouabain. In a typical experiment the assay conditions would be 0.1 M NaCl–0.01 M KCl–0.003 M $MgCl_2$–0.003 M Tris-ATP–0.03 M Tris (pH 7.0)–0.01 unit/ml of ATPase–20 units/ml of pyruvate kinase–2 units/ml of lactic dehydrogenase–1.5×10^{-4} M NADH–1.5×10^{-3} M phosphoenolpyruvate at 37°. The ionic strength was maintained at 0.3 M with choline chloride. The reaction was followed at 340 mμ.

Kinetics of Ouabain Inhibition. Using the assay method above, various concentrations of ouabain were added and the absorbance at 340 mμ was followed until the enzyme was completely inhibited. A typical set of experiments is illustrated in Figure 1. To obtain the kinetics of ouabain inhibition, the ouabain-insensitive base line was extrapolated back to zero time (the dashed line in Figure 1). If A is the absorbance

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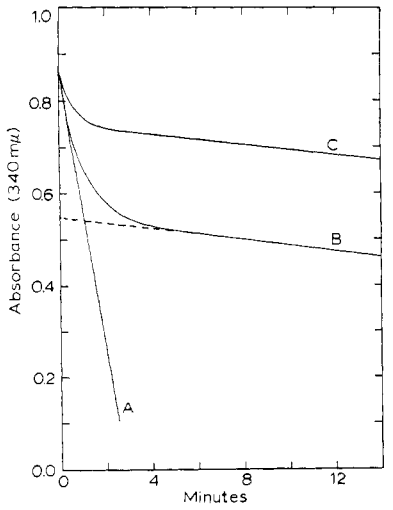

FIGURE 1: A typical set of experiments for determining the rate of ouabain inhibition. The conditions are 0.1 M NaCl–0.01 M KCl–3 mM Tris-ATP–3 mM $MgCl_2$–20 units/ml of pyruvate kinase–2 units/ml of lactic dehydrogenase–0.15 mM NADH–1.5 mM phosphoenolpyruvate, pH 7.0, ionic strength 0.3 M, at 37° and no ouabain (curve A), 3.33 μM ouabain (curve B), and 6.67 μM ouabain (curve C).

at time t at 340 mμ and A_i is the absorbance at time t read from the ouabain-insensitive base line, then ΔA is defined by eq 1. A plot of ΔA *vs.* t is shown in Figure 2.

$$\Delta A = A - A_i \quad (1)$$

Kinetics of Ouabain Binding. The ATPase was incubated with 0.003 M $MgCl_2$–0.003 M Tris-ATP, and various concentrations of NaCl, KCl, and [^3H]ouabain at 37°. The ionic strength was maintained at 0.3 M with choline chloride. At various time intervals 0.5-ml aliquots were taken to which were added 0.05 ml of unlabeled 10^{-3} M ouabain. The enzyme suspension was immediately filtered through a membrane filter, pore size 0.2 μ (obtained from Sartorius, division of Brinkman Instruments, Inc.), and washed twice with distilled water, and the filter dissolved in 10 ml of Bray's solution (Bray, 1960) which was then counted. In a control experiment in which an excess of enzyme over [^3H]ouabain was used, all of the counts were retained on the filter, while if unlabeled 10^{-4} M ouabain was added before the [^3H]ouabain, less than 5% of the counts were retained by the filter.

Results

Kinetics of Ouabain Inhibition. Inhibition of the $(Na^+ + K^+)$ATPase by ouabain is first order if the ouabain concentration is in great excess. Good first-order kinetics are obtained for at least 3 half-lives (Figure 2). The pseudo-first-order rate constant for ouabain inhibition is directly proportional to the ouabain concentration (Figure 3). The kinetics of inhibition are given by eq 2 where k_i is the second-order rate constant for

$$v_i = k_i[E_T][I] \quad (2)$$

inhibition, $[E_T]$ the total enzyme concentration, and [I] the ouabain concentration. The rate constant k_i depends on

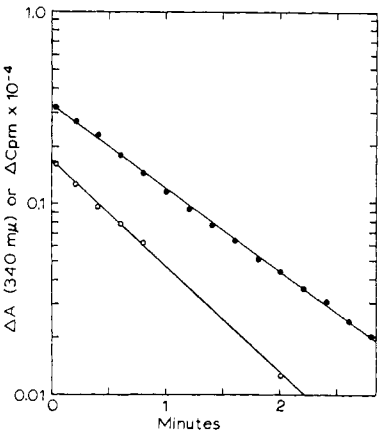

FIGURE 2: Kinetics of ouabain binding and inhibition at 37°, pH 7.0, and ionic strength 0.3 M. Kinetics of binding for 0.5 μM [^3H]-ouabain–3 mM Tris-ATP–3 mM $MgCl_2$–0.1 M NaCl (○); kinetics of inhibition, conditions the same as for Figure 1 with 3.33 μM ouabain (●).

sodium and potassium ion concentrations as is shown in Table I. Complete inhibition of the enzyme occurred with 5×10^{-8} M ouabain, the lowest concentration of ouabain used in the inhibition experiments. Assuming that a 10% difference from complete inhibition could be detected, this sets an upper limit of 5×10^{-9} M for the dissociation constant, K_i, of ouabain under these conditions.

Kinetics of Ouabain Binding. The rate of [^3H]ouabain binding is the same as for inhibition. The rate of ouabain binding is slower in the presence of potassium ion but the final amount bound is the same (Figure 4). Ouabain binding shows good first-order kinetics for at least 3 half-lives (Figure 2); 93 pmoles of ouabain bind per unit of enzyme, where a unit is defined to be the amount of enzyme which hydrolyzes 1 μmole of ATP/min at 37°. Assuming one ouabain per active site this gives a turnover number of 10,800 min^{-1} in the presence of 0.1 M NaCl–0.01 M KCl–0.003 M $MgCl_2$–0.003 M Tris-ATP. This may be compared to values of 8400–16,700 min^{-1} for the $(Na^+ + K^+)$ATPase from 11 species (Bader *et al.*, 1968).

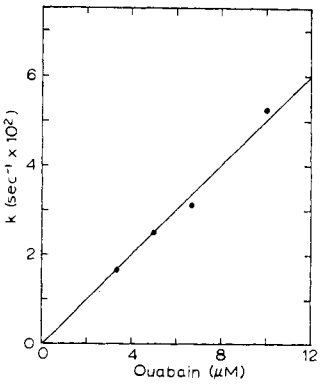

FIGURE 3: Dependence of the pseudo-first-order rate constant for ouabain inhibition on ouabain concentration at 37°, pH 7.0, and ionic strength 0.3 M, with 0.1 M NaCl–0.01 M KCl–3 mM Tris-ATP–3 mM $MgCl_2$.

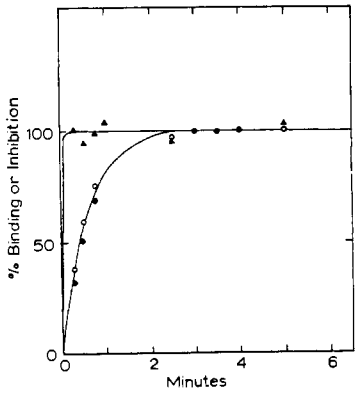

FIGURE 4: Kinetics of ouabain binding and inhibition at 37° and ionic strength 0.3 M. Kinetics of inhibition for 5 μM ouabain, other conditions the same as for Figure 1 (●); kinetics of binding for 5 μM [^3H]-ouabain, conditions the same as for inhibition (○); kinetics of binding, conditions the same as for inhibition except KCl omitted (▲).

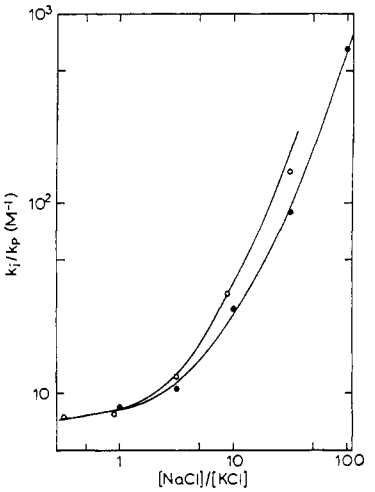

FIGURE 5: Dependence of k_i/k_p at 37°, pH 7.0, and ionic strength 0.3 M on [Na^+]/[K^+] for 0.1 M NaCl (●); 0.03 M NaCl (○). The curves are calculated from eq 3.

The kinetics of ATP hydrolysis were determined for the same conditions as for ouabain inhibition. The results are given in Table I as k_p, the turnover number determined for the same experimental conditions but with ouabain omitted. The data are adequately described by eq 3 (see Figure 5).

$$\frac{k_i}{k_p} = 6.6 + 1.5\frac{[Na^+]}{[K^+]} + 4.58 \times 10^{-3}\frac{[Na^+]}{[K^+]^2} \quad (3)$$

The binding of 10^{-8} M [^3H]ouabain to 3×10^{-8} M ($Na^+ + K^+$)ATPase (the enzyme concentration, with respect to ouabain binding sites, was determined from the binding of excess [^3H]ouabain) was complete in the presence of 0.1 M NaCl–0.003 M $MgCl_2$–0.003 M Tris-ATP. Assuming a 10% error in the determination of the completeness of binding, an upper limit for the dissociation of ouabain for these conditions is 2.3×10^{-9} M. This may be compared with the upper limit of 5×10^{-9} M obtained from the kinetics of ouabain inhibition in the presence of potassium ion. Thus, within the range of ouabain concentrations used in these experiments, potassium ion had no effect on the extent of ouabain binding.

The rate constant for ouabain binding in the presence of 0.1 M NaCl–0.003 M $MgCl_2$–0.003 M Tris-ATP is 4.35×10^4 M^{-1} sec^{-1} (Figure 2). If the dissociation constant is less than 2.3×10^{-9} M, then the rate constant for dissociation of ouabain must be less than 10^{-4} sec^{-1}. Hence the half-life for dissociation must be greater than 1.9 hr.

TABLE I: The Kinetics of ATP Hydrolysis and Ouabain Binding at 37° and Ionic Strength 0.3 M.

[KCl] (M × 10^3)	[NaCl] (M)	k_p[a] (sec^{-1} × 10^{-2})	k_i (M^{-1} sec^{-1} × 10^{-3})	k_i/k_p (M^{-1})
1.00	0.100	0.425	27.6	649
3.33	0.100	1.34	12.0	89.6
10.0	0.100	1.79	4.98	27.8
33.3	0.100	2.32	2.46	10.6
100	0.100	2.16	1.85	8.56
1.00	0.0333	0.787	11.5	146
3.33	0.0333	1.55	5.16	33.3
10.0	0.0333	2.12	2.59	12.2
33.3	0.0333	2.18	1.65	7.59
100	0.0333	1.41	1.06	7.52

[a] Calculated from a turnover number of 1.79×10^2 sec^{-1} determined for 0.10 M NaCl and 0.010 M KCl. See text for details.

Discussion

Comparison of the kinetics of ouabain binding and inhibition demonstrates that (1) the rates of binding and inhibition are identical, (2) the rate of inhibition is first order in both enzyme and ouabain, (3) the dissociation constant for ouabain is less than 5×10^{-9} M, (4) increasing the concentration of potassium ions decreases the rates of binding and inhibition but does not affect extent of binding and inhibition, and (5) increasing the concentration of sodium ions increases the rate of inhibition.

Since the kinetics of inhibition are first order in both ouabain and enzyme one can conclude that the binding of one ouabain per active site inhibits the enzyme. In addition, since the inhibition of the enzyme in the presence of excess ouabain shows good first-order kinetics for at least three half-lives, if there is more than one active site per enzyme then they are probably noninteracting.

There is considerable evidence that the ($Na^+ + K^+$)ATPase involves a phosphorylated intermediate which is probably an acyl phosphate (Post *et al.*, 1965; Hokin *et al.*, 1965; Kahlenberg *et al.*, 1968). Formation of the intermediate is activated by sodium ions while hydrolysis is activated by potassium ions (Post *et al.*, 1965). Activation of the ATPase by sodium and potassium ions is sigmoid (Skou, 1957; Priestland and Whittam, 1968) while for cation transport 3 sodium ions and 2 potassium ions are transported per ATP split (Garrahan

and Glynn, 1967). There appear to be two conformational states of the enzyme, E_1 and E_2. The E_1 state is predominant for the unphosphorylated enzyme while the E_2 state is stabilized by phosphorylation. Conversion of the E_1 phosphorylated state to the E_2 state requires magnesium ion. The E_1 phosphorylated state is dephosphorylated by ADP but not by potassium ion (Post *et al.*, 1969). The E_2 conformation, which is stabilized by phosphorylation, is apparently the form which binds ouabain (Schwartz *et al.*, 1968; Albers *et al.*, 1968). A scheme based on that of Albers (1967) is shown in eq 4–7.

$$E_1 + MgATP + mNa^+ \rightleftharpoons E_1{\sim}P\cdot Na_m + MgADP \quad (4)$$

$$E_1{\sim}P\cdot Na_m \overset{Mg^{2+}}{\rightleftharpoons} E_2{-}P\cdot Na_r + (m-r)Na^+ \quad (5)$$

$$E_2{-}P\cdot Na_r + nK^+ \rightleftharpoons E_2{-}P\cdot K_n + rNa^+ \quad (6)$$

$$E_2{-}P\cdot K_n \longrightarrow E_1 + P_i + nK^+ \quad (7)$$

Although order of action of the monovalent cations in the activation of the ATPase has not been unequivocally established, since phosphorylation requires only sodium ions while dephosphorylation only potassium ions, it seems probable that the scheme of eq 4–7 provides a useful working model for the analysis of kinetic data. Recently further data has been presented supporting this scheme (Post *et al.*, 1969; Sen *et al.*, 1969). No explicit stoichiometry for monovalent cation activation has been assumed, although it is assumed one exists. In eq 5 m may or may not be equal to r.

Potassium ion activation of the ATPase and of cation transport in red blood cells occurs only from the outside of the cell while sodium ion activation of transport and of the ATPase occurs from the inside; ATP is hydrolyzed and orthophosphate is released on the inside, while ouabain acts only from the outside (Whittam, 1962; Whittam and Ager, 1964; Hoffman, 1966). It seems reasonable that for the E_1 conformation the "active site" is more accessible from the inside of the cell while for the E_2 conformation the "active site" is accessible from the outside. Since in the presence of ATP, magnesium ions, and sodium ions the enzyme is probably quantitatively converted into the $E_2{\sim}P$ state, while addition of an optimal concentration of potassium ions gives rapid dephosphorylation to give a barely detectable steady-state level of phosphorylated enzyme (Post *et al.*, 1965), a step leading to $E_2{\sim}P$ is probably rate determining.

For saturating levels of ATP, magnesium ions, and potassium ions, eq 4–7 can be rewritten as eq 8. The intermediates

$$E_1\cdot MgATP\cdot Na_m \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} E_2{-}P\cdot Na_r \rightleftharpoons \dots \rightleftharpoons$$
$$E_2{-}P\cdot Na_xK_y \rightleftharpoons \dots \rightleftharpoons E_2{-}P\cdot K_n \overset{k_c'}{\longrightarrow} E_1\cdot MgATP\cdot Na_m \quad (8)$$

$E_2{-}P\cdot Na_xK_y$ are meant to include states of competitive inhibition between sodium and potassium ions. If one assumes that the steady-state approximation applies to eq 8 and that k_{-c} is negligible then the rate law is given by eq 9–11, where k_p is the turnover number of the enzyme. That k_{-c} is negligible is reasonable since the enzyme appears to be quantitatively phosphorylated in the absence of potassium ions (Bader *et al.*, 1968; Schwartz *et al.*, 1968).

$$k_p = \frac{k_c}{1 + \frac{k_c}{k_c'}F} \quad (9)$$

$$F = 1 + K_{o,n}\sum_{y=0}^{n-1}\sum_{x=0}^{r}\frac{[Na^+]^x[K^+]^y}{K_{x,y}} \quad (10)$$

$$K_{x,y} = \frac{[Na^+]^x[K^+]^y[E_2{-}P]}{[E_2{-}P\cdot Na_xK_y]} \quad (11)$$

Considerable evidence indicates that cardiac glycosides such as ouabain interact preferentially with the phosphorylated enzyme (Post *et al.*, 1965; Fahn *et al.*, 1968; Whittam *et al.*, 1964; Nagano *et al.*, 1967). If the phosphorylated enzyme $E_2{-}P\cdot Na_xK_y$ reacts with ouabain with a second-order rate constant $k_{x,y}$, then the observed rate constant for inhibition, k_i, will be given by eq 12. The empirical equation which

$$\frac{k_i}{k_p} = \frac{k_{o,n}}{k_c'} + \frac{K_{o,n}}{k_c'}\sum_{y=0}^{n-1}\sum_{x=0}^{r}k_{x,y}\frac{[Na^+]^x[K^+]^y}{K_{x,y}} \quad (12)$$

adequately fits the data is given in eq 3. If $x = 1$ and $y = n-2$, $n-1$ in eq 12 with all other terms negligible (this is equivalent to the dissociation constants $K_{x,y}$ being large), then eq 12 corresponds to eq 3 with $k_{o,n}/k_c' = 6.6$, $K_{o,n}k_{1,n-1}/K_{1,n-1}k_c' = 1.5$, and $K_{o,n}k_{1,n-2}/K_{1,n-2}k_c' = 4.58 \times 10^{-3}$. Thus the scheme in eq 8 seems to adequately describe the data for ouabain inhibition if one assumes that ouabain interacts predominately with the phosphorylated enzyme. If eq 8 represents an adequate model for the enzyme then the data imply that the principle forms of $E_2{-}P$ for the experimental conditions of this paper are those with no sodium ions and n potassium ions bound, one sodium ion and $n-1$ potassium ions bound, and one sodium ion and $n-2$ potassium ions bound. If n is the same for transport and for activation of the ATPase, then $n = 2$.

In the context of eq 8 it is possible to answer the question of the effects of monovalent cation liganding on the rate of ouabain binding. If and only if $k_{x,y} = k_i'$, *i.e.*, the rate of ouabain binding to $E_2{-}P$ is independent of cation liganding, eq 12 can be simplified to eq 13. If the assumption that $k_{x,y} =$

$$\frac{k_i}{k_p} = \frac{k_i'}{k_c'}F \quad (13)$$

k_i' is a valid one, then combining eq 3 and 13 gives eq 14 for

$$F = 1 + 0.227\frac{[Na^+]}{[K^+]} + 6.94 \times 10^{-4}\frac{[Na^+]}{[K^+]^2} \quad (14)$$

F and $k_i'/k_c' = 6.6$. This value of F when substituted back into eq 9 should fit the data for the effects of monovalent cations on ATP hydrolysis. To do this a value for k_c and k_c/k_c' is needed. As was mentioned above a step leading to phosphorylation is probably rate determining when optimal cation concentrations are employed. Thus the optimal turnover number for the enzyme should be k_c, which from Table I is 2.32×10^2 sec^{-1}. If the assumption that $k_{x,y} = k_i'$ is correct, then $k_i' = 4.35 \times 10^4$ M^{-1} sec^{-1} from the rate of

ouabain binding to E_2-P in the absence of potassium ions and so $k_o' = 6.59 \times 10^8$ sec^{-1}. Then k_c/k_p, which is equal to V_{max}/V, is given by eq 15. It should be emphasized that eq 15,

$$\frac{V_{max}}{V} = 1.0 + 8.0 \times 10^{-3} \frac{[Na^+]}{[K^+]} + 2.4 \times 10^{-5} \frac{[Na^+]}{[K^+]^2} \quad (15)$$

which was derived from eq 3, 9, and 14, will be valid only if the assumption that $k_{x,y} = k_i'$ is correct. From Table I $K_{0.5}$ for potassium ion is 2.7×10^{-3} M for 0.1 M sodium chloride and 1.6×10^{-3} M for 0.03 M sodium chloride. The calculated values from eq 15 are 2.1×10^{-3} M and 1.1×10^{-3} M, respectively. The agreement is good enough to indicate that the state of monovalent cation liganding to E_2-P has little if any effect on the rate of ouabain binding. Given that the assumption that $k_{x,y} = k_i'$ seems to be valid and that eq 8 provides a working model for the enzyme, the values of k_o, k_c', and k_i' are those given above.

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