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KINETICS OF (Na^+,K^+)-ATPase OF HUMAN ERYTHROCYTE MEMBRANES

II. INHIBITION BY OUABAIN

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SUMMARY

Kinetic studies on the inhibition of (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of human erythrocyte membranes by ouabain have led to the following conclusions:

1. (Na^+,K^+)-ATPase is uncompetitively inhibited by ouabain at concentrations of Na^+ and K^+, which are normally required for optimal activation.

2. Inhibition experiments with ouabain refer to the existence of two types of phosphorylated intermediates, *i.e.* EK^+–P_i and $Na_2^+EK^+$–P_i.

3. Ouabain reacts with both types of phosphorylated intermediates of the enzyme. The affinities between ouabain and EK^+–P_i, and $Na_2^+EK^+$–P_i, respectively, do not differ significantly.

4. The binding of ouabain causes an elimination of K^+ from the phosphorylated intermediates. The relative degree of this ouabain inhibition is closely related to the K^+ concentration: The degree of inhibition is decreased by increasing K^+ concentrations and *vice versa*. In detail, the antagonism between K^+ and ouabain is comparable with a partially competitive type of inhibition.

5. Since EK^+–P_i is not able to split into the end products $EK^+ + P_i$, not only K^+, but also Na^+ is essential for the release of inorganic phosphate from the active phosphorylated intermediate $Na_2^+EK^+$–P_i.

6. The complete reaction mechanism, as derived from experiments with varied concentrations of Na^+, K^+, $MgATP^{2-}$, and ouabain includes the following enzyme intermediates: EK^+, EK^+–$MgATP^{2-}$, $Na_2^+EK^+$, $Na_2^+EK^+$–$MgATP^{2-}$, $Na_2^+EIK^+$–P_i, Na_2^+EI–P_i, EK^+–P_i, EIK^+–P_i, and EI–P_i.

INTRODUCTION

Cardiac glycosides, especially ouabain, are known as potent and specific inhibitors of (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3)[1–3].

Since the inhibition of the (Na^+,K^+)-ATPase coincides with the inhibition of the active Na^+ and K^+ transport[4–6], the elucidation of the reaction mechanism is of great interest. It could be demonstrated with the help of the label technique that ouabain reacts with a phosphorylated intermediate of the ATPase[7–11]. These experi-

ments, however, were not always carried out in the simultaneous presence of Na^+ and K^+.

The dependence of the ouabain inhibition on the K^+ concentration has often been demonstrated[1,12-15]. No general agreement was obtained, however, with respect to the effects of Na^+.

In this paper a mechanism of the ouabain inhibition based on kinetic measurements is suggested. In addition, the function of Na^+ and K^+ in this inhibition mechanism is examined in the simultaneous presence of both Na^+ and K^+.

MATERIALS AND METHODS

Materials and Methods were applied essentially as described in preceding reports[16,17].

DERIVATION OF RATE EQUATIONS

All rate equations are derived according to models presented by Botts and Morales[18], Laidler[19] and Ohlenbusch[20] and to the method reported by Cleland[21], assuming a rapid equilibrium type of reaction, as explained in detail in a previous paper[17]. The rate equations are presented in the reciprocal form throughout.

DEFINITIONS

In addition to the symbols defined in the preceding report[17], the following symbols are used:

I = inhibitor = ouabain

$\bar{k}_{-Na^+}/\bar{k}_{Na^+} = \bar{K}_{Na^+}$ = equilibrium constant of the reaction

$EK^+\text{-}P_i + 2\,Na^+ \rightleftharpoons Na_2{}^+EK^+\text{-}P_i$

$k_{-i}/k_i = K_i$ = equilibrium constant of the reaction

$Na_2{}^+EK^+\text{-}P_i + I \rightleftharpoons Na_2{}^+EIK^+\text{-}P_i$

$\bar{k}_{-i}/k_i = \bar{K}_i$ = equilibrium constant of the reaction

$EK^+\text{-}P_i + I \rightleftharpoons EIK^+\text{-}P_i$

$k'_{-K^+}/k'_{K^+} = K'_{K^+}$ = equilibrium constant of the reaction

$Na_2{}^+EI\text{-}P_i + K^+ \rightleftharpoons Na_2{}^+EIK^+\text{-}P_i$

$k'_{-K^+}/k_{K^+} = \bar{K}'_{K^+}$ = equilibrium constant of the reaction

$EI\text{-}P_i + K^+ \rightleftharpoons EIK^+\text{-}P_i$

$\tilde{V}^I$ = reaction rate at infinite concentration of $MgATP^{2-}$ and any concentration of the inhibitor ouabain

RESULTS

During the kinetic studies of the (Na^+, K^+)-ATPase the following parameters were varied: (1) The $MgATP^{2-}$ and the ouabain concentrations at optimum Na^+ and

K^+ concentrations. (2) The Na^+ and the ouabain concentrations at constant substrate and K^+ concentrations. (3) The K^+ and the ouabain concentrations at constant substrate and Na^+ concentrations.

(1) *Variation of the $MgATP^{2-}$ and the ouabain concentrations*

As shown in Figs 1 and 2, the Lineweaver–Burk[22] and the Dixon plots[23] show parallel, straight lines. This result indicates that the (Na^+, K^+)-ATPase is inhibited uncompetitively by a reaction involving the binding of one molecule ouabain per active centre and that the molar ratio ouabain/substrate is equal to one.

(2) *Variation of the Na^+ and the ouabain concentrations*

The results of these variations are presented in a $1/v$–[ouabain] plot (Dixon[23]) and in a $1/v$ *versus* $1/[Na^+]$ plot (Figs 3 and 4). In both graphs the straight lines intersect

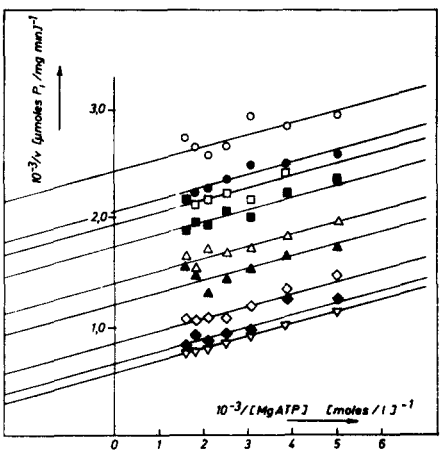


Fig. 1. Plot of $1/v$ *versus* $1/[MgATP^{2-}]$ at various ouabain concentrations. $[Na^+] = 86$ mM, $[K^+] = 8.6$ mM, pH 7.5. [Ouabain]. ▽, 0 μM; ◆, 0.3 μM; ◇, 1.0 μM; ▲, 2.0 μM; △, 3.0 μM; ■, 4.0 μM; □, 4.5 μM, ●, 5.0 μM; ○, 6.3 μM

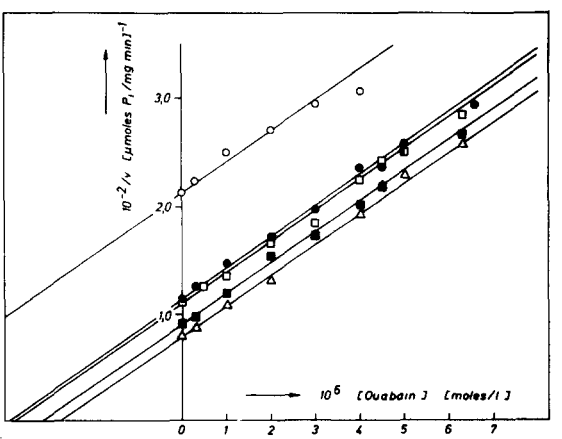


Fig. 2. Plot of $1/v$ *versus* [ouabain] at various $MgATP^{2-}$ concentrations. $[Na^+] = 86$ mM, $[K^+] = 8.6$ mM, pH 7.5. $[MgATP^{2-}]$: △, 0.48 mM; ■, 0.33 mM; □, 0.26 mM; ●, 0.2 mM; ○, 0.07 mM.

at one point on the abscissa. The occurrence of one intersection point in Fig. 3 indicates both that the relative degree of inhibition by ouabain is independent from the Na^+ concentration and that the relative degree of activation by Na^+ is independent from the ouabain concentration (Fig. 4). This means that the dissociation constants of an $Na_2{}^+E$ complex and an E–ouabain complex are independent from the ouabain and the Na^+ concentrations, respectively.

(3) Variation of the K^+ and the ouabain concentration

In contrast to the results obtained with varied Na^+ concentrations the straight lines of the $1/v$–$1/[K^+]$ and $1/v$–[ouabain] plots (Figs 5 and 6) intersect at one point in the second quadrant. This result indicates a competition between K^+ and ouabain. The position of the intersection point refers to a non-exclusive binding of K^+ or ouabain by the (Na^+, K^+)-ATPase, *i.e.* a ternary enzyme–K^+–ouabain complex is

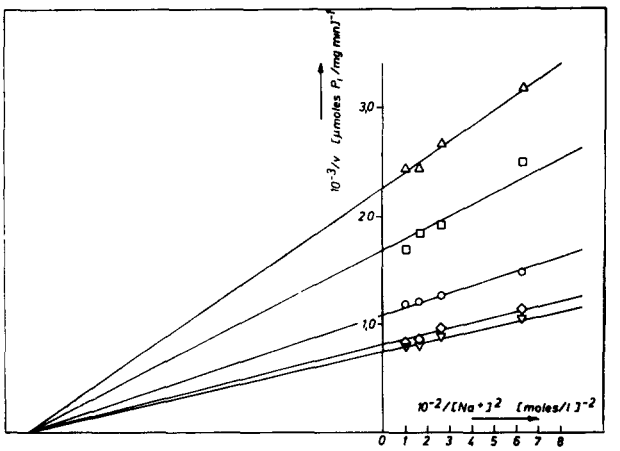


Fig. 3. Plot of $1/v$ *versus* $1/[Na^+]^2$ at various ouabain concentrations. $[K^+]$ = 8.6 mM, $[MgATP^{2-}]$ = 0.48 mM, pH 7.5 [Ouabain] ▽, 0 μM; ◇, 0.3 μM, ○, 1.0 μM; □, 3.0 μM; △, 5.0 μM.

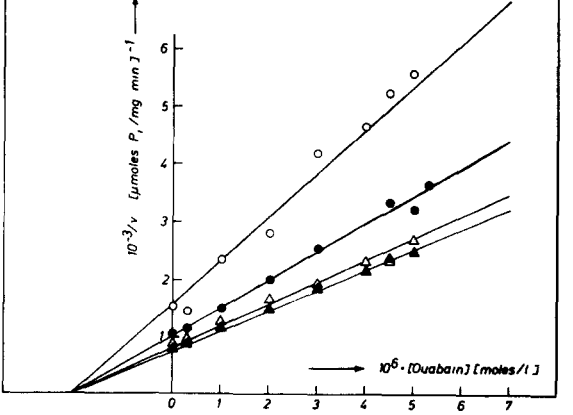


Fig. 4. Plot of $1/v$ *versus* [ouabain] at various Na^+ concentrations. $[K^+]$ = 8.6 mM, $[MgATP^{2-}]$ = 0.48 mM, pH 7.5 $[Na^+]$ = ▲, 80 mM, △, 60 mM, ●, 40 mM; ○, 21 mM.

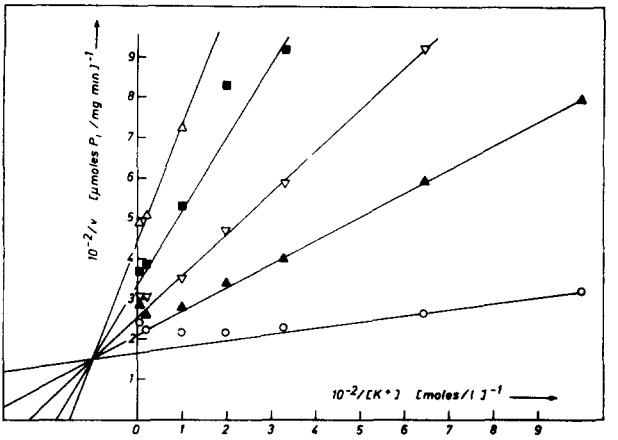


Fig 5 Plot of $1/v$ *versus* $1/[K^+]$ at various ouabain concentrations. $[Na^+]$ = 86 mM, $[MgATP^{2-}]$ = 0.662 mM, pH 7 5 [Ouabain] ○, 0 μM; ▲, 1 0 μM, ▽, 2 0 μM; ■, 4 0 μM; △, 7 0 μM.

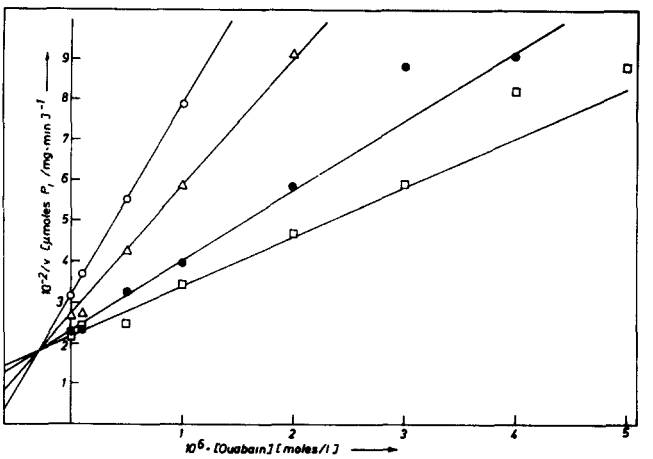


Fig. 6 Plot of $1/v$ *versus* [ouabain] at various K^+ concentrations. $[Na^+]$ = 86 mM, $[MgATP^{2-}]$ = 0 662 mM, pH 7 5 $[K^+]$: □, 1 0 mM, ●, 1 65 mM; △, 3 0 mM, ○, 5 0 mM.

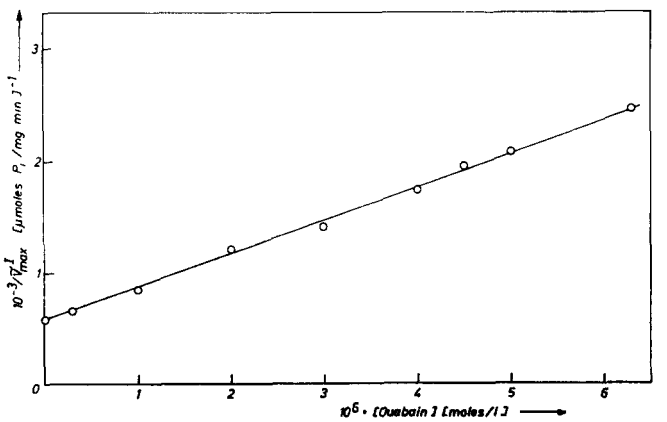


Fig. 7 Plot of the intercepts of Fig. 1 ($\tilde{V}^I$) *versus* [ouabain] in order to evaluate the apparent constant of the ouabain inhibition.

existent. This type of competition is comparable with a partial competitive type of inhibition.

On the basis of the activation mechanism of (Na^+, K^+)-ATPase by Na^+ and K^+, as presented in a previous paper[17], the mode of inhibition and the point of attack by ouabain can be derived as outlined below:

According to Figs 1 and 2, (Na^+, K^+)-ATPase is inhibited uncompetitively, one molecule ouabain being bound per active centre of the enzyme. The uncompetitive inhibition is characterized by the existence of either one or more enzyme–substrate–inhibitor complexes or enzyme–P_i–inhibitor complexes. Including the complexes between the phosphorylated intermediates and ouabain and considering the competition between K^+ and ouabain (*cf.* Figs 5 and 6), the following equilibria can be postulated:

$$E\mathrm{K}^+\text{–MgATP}^{2-} + I \rightleftharpoons EI\text{–MgATP}^{2-} + \mathrm{K}^+ \quad (1)$$

$$\mathrm{Na_2}^+E\mathrm{K}^+\text{–MgATP}^{2-} + I \rightleftharpoons \mathrm{Na_2}^+EI\text{–MgATP}^{2-} + \mathrm{K}^+ \quad (2)$$

$$\mathrm{Na_2}^+E\mathrm{K}^+\text{–P}_\mathrm{i} + I \rightleftharpoons \mathrm{Na_2}^+EI\text{–P}_\mathrm{i} + \mathrm{K}^+ \quad (3)$$

However, Equilibria 1 and 2 can be neglected in the overall reaction sequence for the following two reasons: (1) Various authors could demonstrate that the phosphorylated intermediate is inhibited by ouabain[7–11]. (2) Our own results obtained previously[17] indicate that a K^+-free E–$MgATP^{2-}$ complex is not existent.

If Equilibrium 3 is included into the reaction scheme, the resulting rate equation

$$\frac{1}{v} = \frac{\frac{k_2}{k_1}\left(1 + \frac{K_s}{\overline{K}_s}\frac{K_{Na^+}}{[Na^+]^2}\right) + 1 + \frac{[I]}{K_i}\frac{K'_{K^+}}{[K^+]}}{V} + \frac{K_s\frac{k_2}{k_1}\left[1 + \frac{K_{Na}}{[Na^+]^2}\left(1 + \frac{K_{K^+}}{[K^+]}\right)\right]}{V}\cdot\frac{1}{[S]} \quad (4)$$

neither fits the results of Fig. 3 nor of Fig. 5. The rate equation (4) does not yield an intercept of all straight lines in a $1/v$ *versus* $1/[Na^+]^2$ plot, as obtained in Fig. 3, and Fig. 5 does not show a joint intercept at the ordinate, as predicted by Eqn 4.

A partial competitivity between K^+ and ouabain

$$\mathrm{Na_2}^+E\mathrm{K}^+\text{–P}_\mathrm{i} + I \rightleftharpoons \mathrm{Na_2}^+EI\mathrm{K}^+\text{–P}_\mathrm{i} \rightleftharpoons \mathrm{Na_2}^+EI\text{–P}_\mathrm{i} + \mathrm{K}^+ \quad (5)$$

as suggested from Figs 5 and 6, yields a rate equation

$$\frac{1}{v} = \frac{\frac{k_2}{k_1}\left(1 + \frac{K_s}{\overline{K}_s}\frac{K_{Na^+}}{[Na^+]^2}\right) + \left[1 + \frac{[I]}{K_i}\left(1 + \frac{K'_{K^+}}{[K^+]}\right)\right]}{V} + \frac{K_s\frac{k_2}{k_1}\left[1 + \frac{K_{Na^+}}{[Na^+]^2}\left(1 + \frac{K_{K^+}}{[K^+]}\right)\right]}{V}\cdot\frac{1}{[S]} \quad (6)$$

which fits the results of Fig. 5, but still not those of Fig. 3. Figs 3 and 4 show that the ouabain inhibition is dependent on the Na^+ concentration, and the Na^+ activation is dependent on the ouabain concentration, respectively. This indicates that another enzyme intermediate, whose composition differs from $Na_2{}^+EK^+\text{–}P_i$ by the absence of Na^+, is also inhibited by ouabain. It is reasonable that this complex will be $EK^+\text{–}P_i$. Indeed, only the involvement of the equilibria derived from this intermediate, *i.e.*

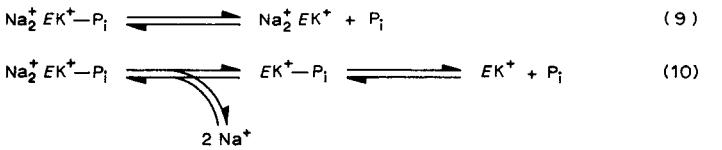
$$Na_2{}^+EK^+\text{–}P_i \rightleftharpoons EK^+\text{–}P_i + 2\,Na^+ \qquad (7)$$

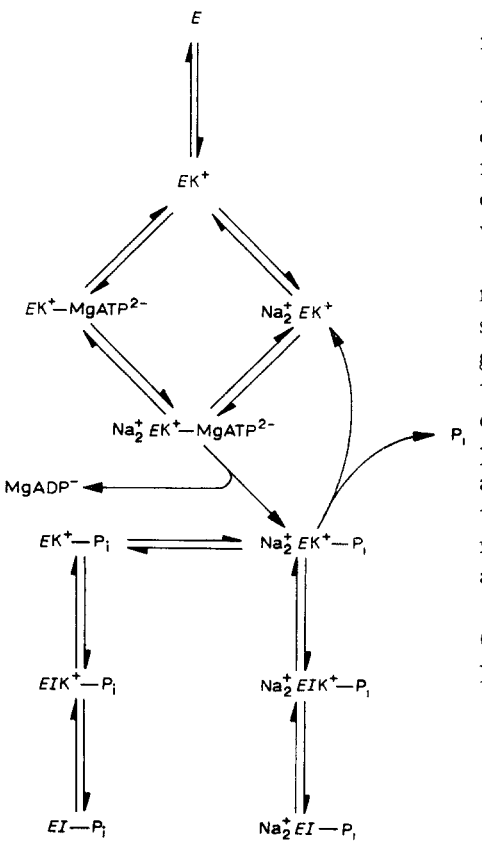
and

$$EK^+\text{–}P_i + I \rightleftharpoons EIK^+\text{–}P_i \rightleftharpoons EI\text{–}P_i + K^+ \qquad (8)$$

combined with the equilibria (5) yield a rate equation, which is in accordance to all experimental results presented in this paper.

Two reactions can be postulated for the decay of the active $Na_2{}^+EK^+\text{–}P_i$ complex:





Only Reaction 9 will occur in the overall reaction, since the involvement of Reaction 10 leads to an additional $[Na^+]^2/K_{Na^+}$ term in the numerator of the $[I]$-free term of any rate equation, indicating that the intercepts of a $1/v$ *versus* $[I]$ plot will increase with increasing Na^+ concentrations, which is in contrast to the results of Fig. 4.

Reaction 9 has to be considered as reversible, since it was possible to demonstrate an incorporation of labelled inorganic phosphate into ATP by reversal of the Na^+ pump in erythrocytes[24,25]. However, under the conditions used in our experiments (measurement of the initial reaction rate, $[P_i] \approx 0$), the backward reaction of (9) is negligible and, therefore, was not taken into account in the following reaction scheme (left):

Complete reaction scheme of the (Na^+, K^+)-ATPase in the simultaneous presence of Na^+, K^+ and ouabain.

Two features of this scheme are of particular interest: (1) Not only K^+, but also Na^+ has to be present in the phosphorylated intermediate to catalyze its decay into the end products. (2) Ouabain attacks exclusively two phosphorylated intermediates, which differ with respect to their Na^+ content, *i.e.* EK^+–P_1 and $Na_2{}^+EK^+$–P_1.

The reaction scheme presented above yields the following rate equation

$$\frac{1}{v} = \frac{\frac{k_2}{k_1}\left(1 + \frac{K_s}{\bar{K}_s}\frac{K_{Na^+}}{[Na^+]^2}\right) + 1 + \frac{[I]}{K_i}\left(1 + \frac{K'_{K^+}}{[K^+]}\right) + \frac{\bar{K}_{Na^+}}{[Na^+]^2}\left[1 + \frac{[I]}{\bar{K}_i}\left(1 + \frac{\bar{K}'_{K^+}}{[K^+]}\right)\right]}{V}$$

$$+ \frac{K_s\frac{k_2}{k_1}\left[1 + \frac{K_{Na^+}}{[Na^+]^2}\left(1 + \frac{K_{K^+}}{[K^+]}\right)\right]}{V} \cdot \frac{1}{[S]} \qquad (11)$$

The rate equation (11), and hence the reaction scheme presented above, is completely in accordance with all characteristics of Figs 1–6. However, in the case of Figs 3 and 4, two conditions have to be fulfilled for this accordance: (1) $K_i \approx \bar{K}_i$ and $K'_{K^+} \approx \bar{K}'_{K^+}$, *i.e.* these constants are not dependent on the absence or presence of Na^+ in the phosphorylated intermediates. (2) $k_2/k_1 \ll 1$, which means that the dephosphorylation of the phosphorylated intermediate is the rate limiting step of the overall reaction.

The numerical values of the equilibrium and reaction rate constants of Eqn 11, cannot be evaluated from results obtained by steady-state kinetics. This problem may be demonstrated, *e.g.* by the following evaluation of ouabain inhibition constants:

The limit of the reaction rate at $1/[S] \to 0$ accounts to

$$\lim_{(1/[S]\to 0)} 1/v = \frac{\frac{k_2}{k_1}\left(1 + \frac{K_s}{\bar{K}_s}\frac{K_{Na^+}}{[Na^+]^2}\right) + 1 + \frac{\bar{K}_{Na^+}}{[Na^+]^2}}{V}$$

$$+ \frac{\frac{1}{K_i}\left(1 + \frac{K'_{K^+}}{[K^+]}\right) + \frac{1}{\bar{K}_i}\left(1 + \frac{\bar{K}'_{K^+}}{[K^+]}\right)\frac{\bar{K}_{Na^+}}{[Na^+]^2}}{V} \cdot [I] \qquad (12)$$

Thus, the plot of the intercepts of Fig. 1 *versus* the ouabain concentration yields a straight line with the slope of

$$\frac{d(\lim_{(1/[S]\to 0)} 1/v)}{d[I]} = \frac{\frac{1}{K_i}\left(1 + \frac{K'_{K^+}}{[K^+]}\right) + \frac{1}{\bar{K}_i}\left(1 + \frac{\bar{K}'_{K^+}}{[K^+]}\right)\frac{\bar{K}_{Na^+}}{[Na^+]^2}}{V} \qquad (13)$$

as shown in Fig. 7. From this slope, only an apparent inhibition constant is available. This constant is still dependent on the Na^+ and K^+ concentration and on the equi-

librium constants K'_{K^+}, $\bar{K}'_{K^+}$ and $\bar{K}_{Na^+}$. Since these constants cannot be obtained at present, the evaluation of the true inhibition constants K_i and $\bar{K}_i$ is thus impossible. Certainly this problem has to be solved by presteady-state measurements.

DISCUSSION

The data presented in Figs 1 and 2 suggest that the (Na^+, K^+)-ATPase is inhibited uncompetitively by ouabain. One molecule ouabain combines with one active centre of the enzyme in a reversible reaction. This conclusion is in agreement with the results of Hansen[26] and Barnett[27]. Since the plots are linear, it can be excluded that the ouabain-containing complexes decompose to a measurable extent, *i.e.* $\beta = 0$ according to the terminology of Webb[28].

The interpretation of an uncompetitive inhibition often includes some uncertainty concerning the composition of the complex, which is attacked by the inhibitor. Often several possibilities can be discussed: the inhibitor may combine with the activated enzyme–substrate complex and/or with an intermediate. With respect to the (Na^+, K^+)-ATPase the results indicate that ouabain combines exclusively with the K^+- and the (Na^+, K^+)-containing phosphoryl-enzyme. As shown in Figs 3 and 4, the $1/v$ *versus* $1/[Na^+]^2$ and the $1/v$ *versus* [ouabain] plots yield one point of intersection on the abscissa. According to Eqn 11, such an intersection point occurs only, if $K_i \approx \bar{K}_i$ and $K'_{K^+} \approx \bar{K}'_{K^+}$, *i.e.* if–in contrast to the apparent constants–the true dissociation constants of ouabain and K^+ are independent from the binding of Na^+ to the phosphorylated intermediate. This means that the presence of Na^+ has no detectable effect upon the affinity of ouabain and K^+ to the enzyme.

While the relative degree of the ouabain inhibition is independent from the Na^+ concentration, there is a marked competition between K^+ and ouabain in binding to the enzyme[29,30]. This result does not indicate, however, that these effectors compete for the same site of the enzyme, inasmuch as their structure is extremely different. The general understanding of competition implicates that an effector B cannot combine with an enzyme, when an effector A is already bound and *vice versa*. This exclusion of a simultaneous binding of two effectors may occur in spite of their structural differences and/or their different binding sites In contrast to a simple competitivity, in which two effectors compete for the same site, the phenomenon of a general competitivity and of a noncompetitivity as well imply that conformational changes of the enzyme are caused by at least one of the effectors[31]. The occurrence of competitivity as a result of a conformational change is supported by the finding of a partial competitivity, which involves the existence of a ternary complex. The formation of a ternary complex is not required in the case of simple competitivity. Thus, the partial competitivity between K^+ and ouabain supports the assumption that K^+ induces a conformational change of (Na^+, K^+)-ATPase (*cf.* Robinson[32]), which prevents to a certain extent the binding of ouabain and *vice versa*.

The finding of a partial competition between ouabain and K^+ is in a certain contrast to the findings of Schatzmann[14] and Matsui and Schwartz[15], who found a noncompetitive interaction between K^+ and ouabain.

A most interesting feature of the ouabain inhibition is that a K^+, which is essential for both the binding of the substrate and the decomposition of the phosphorylated intermediate, is eliminated from the enzyme complex as the result of a

conformational change. The loss in K^+ must result in a marked increase of the stability of the phosphorylated enzyme, inasmuch as the ouabain-containing complex shows no measurable hydrolysis (see Figs 1 and 2). This result agrees with the finding that the ouabain-containing phosphorylated intermediate is more stable than the ouabain-free intermediate[33].

According to Eqn 13 $1/\tilde{V}^I$ has to be plotted *versus* ouabain in order to evaluate the respective inhibition constant. This plot, however, yields only an apparent constant of the enzyme–ouabain complex, which is still dependent on the Na^+ and the K^+ concentrations (*cf.* Schatzmann[14] and Matsui and Schwartz[15]). We therefore suggest that the K_i values so far presented should be reevaluated with respect to the effects produced by Na^+ and K^+. The differences between K_i values reported for (Na^+, K^+)-ATPases from different sources[34–41] may be due not only to a different affinity between ouabain and the enzyme, but also to different affinities between K^+ and/or Na^+ and the enzyme.

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