Factors influencing the onset of ouabain inhibition of Na,K-ATPase from guinea-pig myocardium

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1 The onset of ouabain inhibition was quantified by analysis with an integrated rate equation from experiments in which the activity of Na,K-ATPase from guinea-pig myocardium had been altered with adenosine 5'-triphosphate (ATP, 0.3–9 mmol l⁻¹) in the absence and presence of a detergent.

2 Under control conditions with increasing ouabain (0.1–100 µmol l⁻¹) and ATP (0.3–1 mmol l⁻¹) concentrations, inhibition developed faster. The acceleration by ouabain became less effective at saturating concentrations leading to a non-linear relationship between pseudo-first-order rate constants of inhibition and ouabain concentration. With a rise of ATP to 3 and 9 mmol l⁻¹, i.e., near total Mg concentration (5 mmol l⁻¹), inhibition was retarded presumably because the free concentrations of Mg and uncomplexed ATP changed. Varying the ATP concentration had little effect on ouabain potency at steady state; Hill coefficients were < 1.

3 The detergent alamethicin (23 µg ml⁻¹) neither interfered with Na,K-ATPase activity nor with inhibition at steady state but accelerated its onset. This supports a role for a lipid barrier in the development of inhibition.

4 While the reaction of low concentrations of ouabain with the receptors seemed to govern inhibition rate, with an increase in steroid concentration in the presence of alamethicin, ATP-dependent enzyme activity interfered with the onset of inhibition. The transition of the enzyme between ouabain-sensitive and ATP-hydrolytic conformations consequently causes the non-linear concentration-dependence of pseudo-first-order rate constants. As the Hill coefficient was < 1, a reaction of ouabain with two receptors also could have contributed to the special concentration-dependence of inhibition rates.

Introduction

Ouabain binding to Na,K-ATPase has been shown to depend on phosphorylation (Matsui & Schwartz, 1968; Post *et al.*, 1969) and to be altered by interventions that modulate enzyme activity, e.g., different cation concentrations or hormones (Clausen & Hansen, 1977; Joiner & Lauf, 1978). From these findings a role of enzyme activity in the onset of inhibition by ouabain may be inferred. In view of the different conformations which hydrolyze adenosine 5'-triphosphate (ATP) and bind ouabain (for review see Skou, 1988) a delay in the onset of inhibition should reflect both the concentration and generation of the ATP-hydrolyzing conformation and its transition to the ouabain-sensitive conformation. One might suppose, if all enzyme molecules are not immediately accessible to ouabain because a fraction is in a ouabain-insensitive conformational state, that the rates of formation and breakdown of ouabain-sensitive and ATP-hydrolyzing conformations may interfere with the rate of inhibition. With an increase in ATP concentration, the activation of hydrolysis via low- and high-affinity ATP binding sites (Karlsh *et al.*, 1978) could enhance the rate of appearance of the ouabain-sensitive conformation. Superimposed on the reaction of ouabain with its receptors, especially at high concentrations when the steroid instantaneously reacts with all available receptors, the conformational transitions of the enzyme could govern inhibition rate.

In contrast to binding kinetics (for review see Hansen, 1984) inhibition of Na,K-ATPase by cardioactive steroids with special reference to the pre-steady state has been given little attention, as there are comparably few studies on the on- or offset of the steroid effect (e.g., Albers *et al.*, 1968; Skou *et al.*, 1971; Yoda, 1973; Yoda *et al.*, 1973; Yoda & Yoda, 1973; Clark *et al.*, 1975). However, knowledge of the factors which interfere with the pre-steady state of the reaction of the steroids with the receptors seems to be desirable, with regard to the mechanism of enzyme action as well as the validity of parameters estimated for the reaction of the steroid with receptors. Since fast reactions are inaccessible to the discontin-

uous sampling protocol in binding experiments, the continuous measurement of the pre-steady-state kinetics of inhibition would be preferable.

Continuous recordings of the onset of inhibition over a broad range of inhibitor concentrations, at the different rates of hydrolysis associated with different ATP concentrations, seem to be missing, in particular under those conditions when enzyme activity is proposed to be limiting. In order to quantify the onset of ouabain inhibition of Na,K-ATPase, a special integrated rate equation was developed that enables the estimation of the pertinent parameters independent of enzyme decay during the experiment.

Methods

Preparation of Na,K-ATPase

The enzyme was prepared as described previously with minor modifications (Halbach *et al.*, 1981; Ebner & Schönsteiner, 1985). In brief, immediately after excision, the hearts of five guinea-pigs (150–200 g) were cooled in ice-cold solution of 0.75 mol l⁻¹ KCl and 5 mmol l⁻¹ histidine. The connective tissue was removed and the ventricles (ca. 3 g) were minced in 40 ml KCl-histidine buffer with a Waring Blender 8011 C at full speed (15 s), homogenized (Potter Elvehjem S, 3 strokes at 800 r.p.m.) and passed through four layers of gauze. The filtrate was made up to 80 ml with KCl-histidine buffer, centrifuged at 170g for 4 min to remove the large fragments and at 1000g for 20 min. The pellet was suspended in 30 ml of 10 mmol l⁻¹ histidine, 0.25 mol l⁻¹ sucrose, homogenized with 3 strokes and further diluted to 80 ml. After centrifugation at 1000g for 20 min the pellet was resuspended in 10 ml of 40% (w/w) sucrose, 10 mmol l⁻¹ histidine, made up to 80 ml with 10 mmol l⁻¹ histidine, and sedimented at 1000g for 20 min. To extract the contractile proteins in the presence of 5 ml of 10 mmol l⁻¹ histidine, with 0.4 mol l⁻¹ LiBr, a concentration of 2 mg protein ml⁻¹ LiBr-histidine solution was prepared and the suspension stirred for 1 h. After the addition of 40 ml

histidine (10 mmol l^{-1}), the cell fragments were spun down ($1000g$, 25 min), washed twice, at first in 20 ml histidine solution, then in 9 ml , at $1000g$. The preparation was kept for use in 1 ml of 10 mmol l^{-1} histidine. pH of all solutions was 7.4 ; temperature was 0°C . Protein was determined according to Lowry *et al.* (1951).

Na,K-ATPase assay

Na,K-ATPase activity was determined with the coupled enzyme test (pyruvate kinase-lactate dehydrogenase, PK/LDH). In the cuvette the solution was vigorously stirred and kept at 35°C ; pH was 7.4 . The reaction medium contained in a final volume of 1.3 ml (in mmol l^{-1}): KCl 5.9 , NaCl 140 , MgCl_2 5 , NaN_3 5 and EGTA 0.5 (to block mitochondrial and Ca-ATPases, respectively), Tris 50 , Tris-ATP 3 , phosphoenolpyruvate 0.5 , NADH 0.24 , $(\text{NH}_4)_2\text{SO}_4$ 16.8 , pyruvate kinase 7 u and lactate dehydrogenase 10 u as PK/LDH mixture, i.e., in several thousand fold excess over ATPase activity; four fold increase of PK/LDH concentration did not affect the activity. After 15 min preincubation, Na,K-ATPase was added ($5\text{--}25 \mu\text{g}$ protein in $50 \mu\text{l}$ histidine, 10 mmol l^{-1}); enzyme activity depended linearly on protein concentration. After a further 10 min , water (control) or ouabain was injected in a volume of $5 \mu\text{l}$ and the time course was followed for $15\text{--}30 \text{ min}$. Mixing was complete within less than 1 s . NADH absorbance was monitored at 334 nm . Absorbance values were transmitted to the computer at 1 or 2 s intervals via an RS 232 interface.

[^3H]-ouabain binding to Na,K-ATPase

[^3H]-ouabain binding was determined under the conditions of the enzyme assay at 35°C with ATP-regenerating enzymes in an incubation volume of 2.6 ml ; additionally $1.66 \mu\text{Ci}$ [^3H]-ouabain (corresponding to a concentration of 28.8 nmol l^{-1}), and unlabelled ouabain were injected. Before the addition of [^3H]-ouabain, $0.166 \mu\text{Ci}$, [^{14}C]-sorbitol was applied for the estimation of non-specific ouabain binding. [^{14}C]-sorbitol space was identical to [^3H]-ouabain space in the presence of $100 \mu\text{mol l}^{-1}$ ouabain. Protein concentration was between 0.22 and 0.33 mg per 2.6 ml incubation volume. After different incubation times aliquots ($100 \mu\text{l}$) were drawn, rapidly filtered over Whatman GF/C glass fibre filters and washed with 1 ml of ice-cold incubation medium. The double-label technique did not necessitate complete wash-out of non-specifically bound ouabain. Partial reduction was sufficient to calculate non-specific [^3H]-ouabain binding via the [^{14}C]-sorbitol content of each sample (see below). The samples were counted by liquid scintillation, 6 to 8 h after 10 ml of scintillation cocktail HP had been added. Medium samples ($50 \mu\text{l}$) were also counted in HP.

An integrated rate equation of enzyme inhibition

During the assay, Na,K-ATPase activity deteriorated by $5\text{--}15\%$ during 15 min incubation. In the presence of 0.1 mmol l^{-1} lactate or of 0.1 mmol l^{-1} Na_2HPO_4 the activity was not different from the control, therefore product inhibition can be excluded. A comparison with the activity of aliquots which had been incubated under similar conditions for different periods but without substrates (no ATP, phosphoenolpyruvate, NADH) showed that 'ageing' of Na,K-ATPase was responsible for the reduction of enzyme activity during the assay. If only steady-state values of inhibition were compared after constant incubation the decay of activity was negligible. However, for the pre-steady state of inhibition it was found that the baseline change of activity interfered with and even precluded its quantification. Therefore it was desirable to estimate the parameters of pre-steady-state and steady-state inhibition independently from enzyme decay.

In the control situation the turn-over of substrate S i.e. $(\text{d}S/\text{d}t)_c$ follows (constant k) enzyme concentration E . If E decays with some function $f(t)$ which for instance could be monoexponential, we obtain

$$-(\text{d}S/\text{d}t)_c = kEf(t) \quad (1)$$

To approximate the time course of inhibition with a pseudo-first-order rate constant (k_1), the time course of the inactive enzyme EI_i generated by the reaction with inhibitor I is set to

$$\text{EI}_i = \text{EI}_\infty(1 - \exp(-k_1t)) \quad (2)$$

where EI_∞ is the equilibrium value of EI_i . In the presence of the inhibitor the active enzyme ($E - \text{EI}_i$) now decays with $f(t)$. The rate equation of substrate turnover $(\text{d}S/\text{d}t)_i$ corresponding to equation 1 then reads

$$-(\text{d}S/\text{d}t)_i = k(E - \text{EI}_\infty(1 - \exp(-k_1t)))f(t) \quad (3)$$

Integration of equation 3 after a function $f(t)$ had been chosen the integrated rate equation and fitting to the time courses of substrate in the absence and presence of the inhibitor would provide the pertinent rate constants. This procedure, however, requires the appropriate function $f(t)$ and long duration of the experiment to obtain information on the decay. Lack of substrate or product inhibition precludes this approach. However, selection of an explicit function $f(t)$ and estimation of the pertinent parameters can be avoided when we consider the properties of equations 1 and 3. Their combination gives

$$(\text{d}S/\text{d}t)_i = (1 - \text{EI}_\infty/E(1 - \exp(-k_1t))) (\text{d}S/\text{d}t)_c \quad (4)$$

Equation 4 transforms the slope of any control curve to that in the presence of the inhibitor during the onset of its effect independent of enzyme decay. When we can describe the slope of the control accurately enough without mechanistic meaning, the parameters of inhibition can be estimated.

The flexibility of polynomials allows their adjustment to a large number of functions. Actually cubic polynomials

$$S = S_0 + bt + ct^2 + dt^3 \quad (5)$$

could be fitted with considerable precision to the control curves ($S_0 = S$ at $t = 0$). A quadratic expression would have served equally well. Substitution of $(\text{d}S/\text{d}t)_c$ in equation 4 with the differentiated equation 5 and integration provides the integrated rate equation which determines the time course of S during the onset of inhibition independent of enzyme decay

$$\begin{aligned} S = S_0 + (1 - \text{EI}_\infty/E)(bt + ct^2 + dt^3) \\ - \exp(-k_1t)\text{EI}_\infty/E \\ \times (b/k_1 + 2c/k_1^2(k_1t + 1) \\ + 3d(t^2/k_1 + 2t/k_1^2 + 2/k_1^3)) \\ + \text{EI}_\infty/E(b/k_1 + 2c/k_1^2 + 6d/k_1^3) \end{aligned} \quad (6)$$

Equation 6 will automatically be reduced to the linear case by setting c and $d = 0$ when enzyme decay is absent.

Evaluation of experiments

The fitting of equation 6 to experimental data (Gauss-Newton method) provided estimates of pseudo-first-order rate constant k_1 and fractional inhibition $\text{EI}_\infty E^{-1}$. Enzyme activity was determined by differentiation of a cubic polynomial fitted to the time course of absorbance (equation 5; for $t = 0$ $\text{d}S/\text{d}t = b$). Half-maximal inhibitory concentrations (IC_{50}) and slope P' (= Hill coefficient) of concentration-inhibition curves at steady state were calculated by fitting a Hill-type equation

$$(\text{EI}_\infty E^{-1} = \text{MIP}'(I^{P'} + \text{IC}_{50}^{P'})^{-1} \quad (7)$$

to experimental data; M = maximal inhibition, I = inhibitor concentration. Confidence limits of estimates were calculated from standard deviation $\times t$ value. Linear regressions were computed by the method of least squares.

Tissue:medium ratios of specific [^3H]-ouabain binding were obtained from $(\text{d.p.m.}_{\text{spec. ouabain filter}} \text{mg}^{-1} \text{ protein of sample}) (\text{d.p.m.}_{\text{ouabain medium}} \mu\text{l}^{-1} \text{ medium sample})^{-1}$ after the total amount of [^3H]-ouabain in the filter had been corrected for that amount of ouabain corresponding to [^{14}C]-sorbitol space; $\text{d.p.m.}_{\text{spec. ouabain filter}} = \text{d.p.m.}_{\text{total ouabain filter}} - \text{d.p.m.}_{\text{sorbitol filter}} (\text{d.p.m.}_{\text{ouabain medium}} / \text{d.p.m.}_{\text{sorbitol medium}})$; d.p.m. disintegrations per min. The data from single experiments or arithmetic means $\pm$ s.e.mean, if not stated otherwise, are shown.

Materials

The suppliers of chemicals were: NaN_3 , Tris-(hydroxymethyl)aminomethane, L-histidine, $\text{Na}_2\text{-ATP}$ (Merck, Darmstadt, Germany); NADH (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt), ouabain (Boehringer, Mannheim, Germany); lactate dehydrogenase/pyruvate kinase mixture in 2.2 mol l^{-1} ammonium sulphate, ethylene glycol bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), alamethicin (Sigma, München, Germany); Dowex 50W $\times$ 8 200–400 mesh (H^+), Na-dodecylsulphate (Serva, Heidelberg, Germany); [^3H]-ouabain (specific activity $22.1 \text{ Ci mmol}^{-1}$) in ethanol:benzene = 9:1; [^{14}C]-sorbitol (specific activity $346 \text{ mCi mmol}^{-1}$) in ethanol:water = 9:1 (New England Nuclear, Dreieich, Germany); scintillation cocktail HP (Beckman, München, Germany).

Tris-ATP was prepared from $\text{Na}_2\text{-ATP}$ with Dowex 50 $\times$ 8.

Results

Ouabain inhibition of Na,K-ATPase at different ATP concentrations

Original data for Na,K-ATPase activity under control conditions and after the addition of $100 \mu\text{mol l}^{-1}$ ouabain using the superimposed fit of equation 6 demonstrated the inhibitory effect of the cardioactive steroid (Figure 1). The initial curvature of the recording with ouabain reflects the onset of inhibition before the effect approaches its steady state. In comparable experiments at different ouabain and ATP con-

Figure 1 The time course of Na,K-ATPase inhibition by ouabain. The onset of Na,K-ATPase inhibition by $100 \mu\text{mol l}^{-1}$ ouabain is shown in comparison with the control (original recordings). The ATP concentration was 3 mmol l^{-1} , temperature 35°C . The curves obtained by fitting equation 6 to data are superimposed. Parameter estimates of fit: k_1 2.0315, $\text{EI}_{\infty}\text{E}^{-1}$ 0.8981, S_0 (ouabain) 1.0584, S_0 (control) 1.0390, b -0.0271, c 0.0003, d < 0.0001. Abscissa scale: time after the injection of water (control) or ouabain. Ordinate scale: NADH absorbance at 334 nm.

centrations, the onset of inhibition in terms of a pseudo-first-order rate constant and the fractional inhibition at steady state were quantified. The hydrolytic activity in the controls varied between 0.26, 0.37, 0.41 and $0.29 \mu\text{mol mg}^{-1} \text{ protein min}^{-1}$ at 0.3, 1, 3 and 9 mmol l^{-1} ATP, respectively. Although the heart enzyme was one hundred fold less purified than a preparation from renal outer medulla (Fuchs & Ebner, 1989) similar findings with the enzyme of high specific activity preclude a role of enzyme purity.

At 0.3 mmol l^{-1} ATP the onset of inhibition accelerated with the lower ouabain concentrations more than they did near the maximum of inhibition (Figure 2), so that the resulting relationship was nonlinear. The nonlinear dependence of rate constants on inhibitor concentration was preserved when inhibition accelerated parallel to the higher hydrolysis rate at 1 mmol l^{-1} ATP ouabain. A further increase of ATP to 3 and 9 mmol l^{-1} i.e., close to total Mg concentration (5 mmol l^{-1}), must reduce free Mg and increase uncomplexed ATP concentration (cf. Skou *et al.*, 1971). Either effect consequently could have retarded inhibition and made its rate independent of ouabain concentration at 9 mmol l^{-1} ATP. The variation of ATP concentration in these experiments altered fractional inhibition of ATP hydrolysis at steady state by only a small amount (Figure 3). Half-maximal inhibitory concentrations of ouabain (IC_{50}) and the maximal fractional inhibition were only slightly different, although they tended towards lower values with an increase in ATP concentration. Estimates of $\text{IC}_{50} \pm 95\%$ confidence limits with equation 7 were 0.59 ± 0.09 , 0.59 ± 0.20 , 0.37 ± 0.057 , $0.35 \pm 0.24 \mu\text{mol l}^{-1}$ at 0.3, 1, 3, 9 mmol l^{-1} ATP, respectively. These IC_{50} values are one order of magnitude lower than the dissociation constants from the ratio of apparent dissociation over association rates derived from the relationship between rate constants and low ouabain concentrations in Figure 2. The corresponding maximal fractional inhibition data were 0.89 ± 0.026 , 0.92 ± 0.05 , 0.85 ± 0.02 , and 0.75 ± 0.09 . With the exception of 9 mmol l^{-1} ATP the Hill coefficient of inhibition was significantly < 1 , as observed previously (Ebner & Schönsteiner, 1985; 0.78 ± 0.09 , 0.73 ± 0.18 , 0.86 ± 0.10 , 0.88 ± 0.47).

Alamethicin accelerates Na,K-ATPase inhibition by ouabain

Limited access of reactants to enzyme particles could interfere with ATP hydrolysis or inhibition. An effect of an unstirred layer of membrane particles on the onset of inhibition was

Figure 2 The onset of Na,K-ATPase inhibition by ouabain. With pseudo-first order rate constants (in min^{-1} , ordinate scale) the onset of inhibition was quantified at different ouabain (abscissa scale, linear) and ATP concentrations (ATP, 0.3 (Δ), 1.0 ($\blacksquare$), 3.0 ($\bullet$) and 9.0 ($\blacklozenge$) mmol l^{-1}). Arithmetic means with some s.e.mean (vertical lines) of 3–7 experiments are shown. At ouabain concentrations $< 10 \mu\text{mol l}^{-1}$ and at 9 mmol l^{-1} ATP s.e.mean were smaller than symbols.

Figure 3 The inhibition of Na,K-ATPase by ouabain at steady state. Ouabain inhibition at steady state is shown as fractional inhibition (ordinate scale) of total ATP hydrolysis at different ATP concentrations (▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l⁻¹. Ouabain concentration is indicated on abscissa scale (log scale). Arithmetic means are shown; s.e.mean were smaller than symbols.

excluded by the ineffectiveness of raising viscosity with 50 mmol l⁻¹ sucrose in two preparations (k_1 2.39 and 2.2 min⁻¹ vs 2.35 ± 0.12 , $n = 7$, of control at 100 μ mol l⁻¹ ouabain, 3 mmol l⁻¹ ATP). Also lipids could impede the access if the reactants have to permeate membrane vesicles to become effective. This idea was tested with detergents which facilitate the permeation of lipids. The detergent-like peptide alamethicin opens vesicles of enzymes (Jones *et al.*, 1980) and extracts lipids from cell membranes (Bonnafeous *et al.*, 1979). At a concentration of 23 μ g ml⁻¹ it neither interfered with ATP hydrolysis (Figure 4) nor influenced either decay rate or fractional inhibition. At the various ouabain (0.1, 1, 10, 100 μ mol l⁻¹) and ATP concentrations, inhibition at steady state after alamethicin treatment was identical to the controls (regression coefficient 1.016 ± 0.037 , $n = 16$; condition for identity = 1) as were the parameters of the ouabain-inhibition curves (the tendency for IC₅₀ values to be lower with an increase in ATP concentration and Hill coefficients < 1

Figure 4 Na,K-ATPase activity in the presence of alamethicin. In the same preparations the ATP-hydrolytic activity (in μ mol (mg protein min)⁻¹) was determined under control conditions (abscissa scale) and in the presence of alamethicin (ordinate scale). Control data represent the initial activity at $t = 0$; to obtain the data with alamethicin the control activity was multiplied with fractional inhibition or activation under steady-state conditions. Data from individual experiments are shown. The straight line with slope = 1 corresponds to the condition of identity. The various symbols represent the different ATP concentrations (▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l⁻¹.

persisted). In contrast to its ineffectiveness at steady state, alamethicin accelerated the onset of ouabain inhibition 2.3 fold but preserved the typical concentration-dependence with respect to ouabain and ATP (Figure 5). Comparable tests with 0.2 mg ml⁻¹ Na-dodecylsulphate at 35°C at first failed, because the enzyme was rapidly inactivated within 2–3 min. However, when incubated for 30 min at room temperature before the test, the accelerating effect of Na-dodecylsulphate (0.1 mg ml⁻¹) was comparable to that of alamethicin without loss of activity (data not shown).

In the presence of alamethicin the rate constants of inhibition clearly correlated with ATPase activity, as determined by the different ATP concentrations (Figure 6) whereby the influence of enzyme activity on inhibition rate increased progres-

Figure 5 Alamethicin accelerates the onset of ouabain inhibition. Arithmetic means of pseudo-first-order rate constants of the onset of inhibition in min⁻¹ under control conditions (abscissa scale, data from Figure 2) and in the presence of alamethicin (23 μ g ml⁻¹; $n = 3-7$; ordinate scale) were correlated at the different ouabain (0.1, 1, 10, 100 μ mol l⁻¹) and ATP concentrations (▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l⁻¹. The parameters of the correlation of means were: $r = 0.97$, $P < 0.01$; $y = (0.15 \pm 0.23) + (2.34 \pm 0.15)x$. The flatter straight line stands for the identity of data (slope = 1).

Figure 6 Na,K-ATPase activity interferes with the onset of ouabain inhibition. The data from individual preparations of Na,K-ATPase activity (μ mol (mg protein min)⁻¹, abscissa scale) obtained at different ATP concentrations (▲) 0.3, (■) 1.0, (●) 3.0 and (◆) 9.0 mmol l⁻¹, were correlated with pseudo-first-order rate constants at different ouabain concentrations (min⁻¹, ordinate scale; experiments of Figure 5). Regressions: ouabain 1 μ mol l⁻¹, $r = 0.52$, $P < 0.05$, $y = (0.55 \pm 0.27) + (2.57 \pm 1.12)x$; ouabain 10 μ mol l⁻¹, $r = 0.77$, $P < 0.001$, $y = (1.72 \pm 0.50) + (9.63 \pm 2.13)x$; ouabain 100 μ mol l⁻¹, $r = 0.88$, $P < 0.001$, $y = (1.61 \pm 1.42) + (30.82 \pm 5.91)x$.

sively from 1 to 100 $\mu\text{mol l}^{-1}$ ouabain. All data obtained with 9 mmol l^{-1} ATP and 3 mmol l^{-1} ATP, 100 $\mu\text{mol l}^{-1}$ ouabain, were omitted from the plot. With these high ATP concentrations inhibition rate was retarded to a greater degree than would be expected relative to enzyme activity.

[^3H]-ouabain binding to Na,K-ATPase

Under the conditions of the preceding experiments (35°C, 145 mmol l^{-1} Na, 5.9 mmol l^{-1} K, ATP-regenerating enzymes) the time course of [^3H]-ouabain binding (0.03 $\mu\text{mol l}^{-1}$) to Na,K-ATPase was evaluated in the presence of 0, 0.3, and 3 $\mu\text{mol l}^{-1}$ unlabelled ouabain. From these experiments tissue: medium ratios (T:M) of specific [^3H]-ouabain binding, i.e., corrected for that amount of ouabain corresponding to [^{14}C]-sorbitol space, were calculated. T:M = 1 corresponds to 0.03, 0.3 and 3 μmol ouabain mg^{-1} protein at the respective ouabain concentration in $\mu\text{mol l}^{-1}$. With an increase in ouabain concentration, saturation of binding became evident. Accordingly, steady-state T:M values (estimated by fitting a monoexponential time curve to data) declined from 60.7 ± 0.7 to 35.5 ± 0.3 and $6.2 \mu\text{l mg}^{-1}$ at 0.03, 0.3, and 3 $\mu\text{mol l}^{-1}$ ouabain, respectively. At 3 $\mu\text{mol l}^{-1}$ ouabain, specific binding was therefore nearly saturated, being close to [^{14}C]-sorbitol space. With pseudo-first-order rate constants of 0.21 ± 0.02 and $0.29 \pm 0.02 \text{ min}^{-1}$ ($\pm 95\%$ confidence intervals), at 0.03 and 0.3 $\mu\text{mol l}^{-1}$ ouabain, binding developed at a rate similar to enzyme inhibition.

Discussion

Subsequent to the occupation of a single class of receptors with immediate onset of inhibition the effect should develop monoexponentially with linear dependence of pseudo-first-order rate constant k on ouabain concentration I (cf. also Wallick & Schwartz, 1974), where $k = k_{+1}I + k_{-1}$; k_{+1} and k_{-1} are the rate constants of association and dissociation, respectively. At low ouabain concentrations the time course of inhibition actually correlated with [^3H]-ouabain binding and accelerated with an increase in ouabain concentration. However, the difference between IC_{50} values and k_{-1}/k_{+1} and the reduced acceleration of inhibition rate with high ouabain concentrations remain at variance.

Limited access of reactants to the enzyme could have contributed to the nonlinear relationship between inhibition rates and ouabain concentration. Alamethicin actually accelerated inhibition but left enzyme activity and steady-state inhibition unaltered. This suggests that although ouabain had to permeate lipids to gain access to the receptor, its steady-state concentration at the receptor was not limited. Moreover, the nonlinear relationship between inhibition rate and ouabain concentration was preserved in the presence of the detergent. Since the aggregation of ouabain molecules at high concentrations is also unlikely, because of concentration-independent diffusion of ouabain in glass fibre strips (Ebner & Siegl, 1988; unpublished results), different mechanisms seem to be relevant to inhibition rate.

In view of the Hill coefficient being < 1 , two receptors may participate in ouabain inhibition in accordance with previous results (e.g., guinea-pig heart, Ebner & Schönsteiner, 1985; rat heart, Wallick & Schwartz, 1988) and with their demonstration in binding experiments (e.g., ox brain, Taniguchi & Iida, 1972; guinea-pig heart, Godfraind *et al.*, 1980). The reaction of ouabain with two independent receptors implies a linear relationship between the rate constants of two exponentials and ouabain concentration. This relationship is governed by the solutions of quadratic equations in the case of interdependent receptors. That is, in both cases the concentration-dependence of pseudo-first-order rate constants is at least qualitatively similar to that of Figure 2. Although isozymes of Na,K-ATPase in guinea-pig myocardium have not been demonstrated definitely (Ng & Akera, 1987; Sweadner, 1989)

in contrast to other tissues or species (e.g., Sweadner, 1979; Ng & Akera, 1987; Maixent *et al.*, 1987), isozymes and interdependent ouabain receptors could be relevant to the present observations. Different populations of ouabain receptors, however, do not suffice to explain all the experimental findings; in particular the relationship between rate and enzyme activity (Figure 6) must be based on a different mechanism.

According to Sen *et al.* (1969) ouabain reacts with the K-sensitive phosphoprotein $\text{E}_2\text{-P}$ which is transformed to a ouabain-containing dephosphoenzyme in a precursor-product relationship. Studies on phosphorylation (Matsui & Schwartz, 1968; Post *et al.*, 1969) have also suggested its relevance to ouabain binding. While steady-state inhibition (Figure 3) was only marginally influenced by enzyme activity, presumably because the activity could not be varied widely enough (inferred from similar experiments on a highly active enzyme from guinea-pig renal outer medulla, Fuchs & Ebner, 1989), ATP hydrolysis clearly interfered with inhibition rate; i.e., the reaction of ouabain with a definite conformation of the enzyme, $\text{E}_2\text{-P}$, did not immediately become evident. With a simplified model of Na,K-ATPase activity some of the pertinent features of pre-steady-state inhibition could be simulated. The access of the reactants to the enzyme was assumed to be unrestricted, conforming to the experimental situation in the presence of alamethicin. By disregarding various intermediate or phosphorylated states, the inhibitor I is supposed to react with E_2 while ATP is hydrolyzed at E_1 ,

E'_1 was added to the model to interfere at various steps of the reaction sequence. The concentration of E_1 determines ATP hydrolysis by $-\text{dATP}/\text{dt} = k_1\text{E}_1$, where k_1 also contains ATP concentration. The backward reaction was omitted as ADP concentration is kept low by ATP-regenerating enzymes. The time course of E_1 accordingly reflects the onset of inhibition, which is accelerated by the concentration of inhibitor until the transition between enzyme conformations becomes rate-limiting (Figure 7a). The value for the maximal rate constant of inhibition (0.65) was below that of the individual rate constants (1) presumably because E_1 declines less rapidly than it would do in a single reaction. At a given inhibitor concentration (Figure 7b) enzyme activity ($k_1\text{E}_1$) interferes with inhibition rate. Basically this effect does not depend on the mechanism which alters enzyme activity, i.e., variation of the various rate constants, but an increase in the inhibitor concentration augments its impact.

In the presence of Tris in Na-rich media, Na,K-ATPase is kept in a Na-like (E_1 , ADP-sensitive) conformation (Jørgensen, 1975; Jørgensen & Petersen, 1982) while the E_2 (K-sensitive) form is favoured by K (Jørgensen, 1975) in the absence of Tris (Skou & Esmann, 1980). At a given Na:K ratio increasing the ATP concentration also favours E_1 (Skou, 1979). At 100 mmol l^{-1} Na, 37.7 mmol l^{-1} K was necessary for half-maximal reversal of the Na-induced shift of E_2 to E_1 (Skou & Esmann, 1983), therefore E_1 is likely to predominate in our experiments. Hence, delayed onset of inhibition would be conceivable because of the protracted decline of the ATP-hydrolytic conformations after occupation of the ouabain-sensitive conformation by the steroid. This effect becomes increasingly relevant when, with high ouabain concentrations, the conformational transitions of the enzyme cycle interfere with inhibition rate (cf. Figure 7b vs Figure 6). However, as compared with maximal inhibition rates (half-times of approximately 5 s at 1 mmol l^{-1} ATP) the Na-induced conformational transition of Na,K-ATPase with half-times of ms (Karlsh & Yates, 1978; Skou & Esmann, 1983) is fast. Although K-induced transition of E_2 to E_1 with half-times of a few s is slower (Karlsh & Yates, 1978; Skou & Esmann, 1983), submillimolar ATP concentrations accelerate this reac-

Figure 7 Model calculations for the relationship between Na,K-ATPase activity and the onset of ouabain inhibition. (a) The dependence of the onset of inhibition on inhibitor concentration and the effect of enzyme activity. For arbitrarily chosen parameters ($k_1 = 1$, $k_2 = 0$, $k_{3-6} = 1$, $k_7 = 0.1$, $k_8 = 0.01$) and arbitrary units half-times of the reduction of E_1 after the addition of different inhibitor concentrations (abscissa scale) were calculated in numerical solutions of the model and transformed to pseudo-first-order rate constants (ordinate scale). (b) The relationship between enzyme activity and the onset of inhibition. For arbitrarily chosen parameters (same as in (a)) and arbitrary units enzyme activity ($E_1 k_1$, abscissa scale) was altered by varying k_1 (0.1, 1, 10, 100, Δ) and pseudo-first-order rate constants of the reduction of E_1 (ordinate scale) were calculated after the addition of a high (1000) or low (1) inhibitor concentration. For (Δ) enzyme activity was altered by varying k_3 and k_4 (both 0.1, 1, 10, $k_1 = 1$) at an inhibitor concentration = 1000.

tion by nearly two orders of magnitude. The difference between transition and maximal inhibition rates therefore indicates an interference with, but not the complete control of, inhibition rate by the conformational transitions of the enzyme.

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