Kinetic Analysis of the Positive Inotropic Action (PIA) of Ouabain in Isolated Perfused Rabbit Heart. Slow Onset of PIA and Slow Binding to Na⁺, K⁺-Adenosine Triphosphatase

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The positive inotropic action (PIA) of ouabain was analyzed kinetically using isolated perfused rabbit heart. The input function of the ouabain concentration in the perfusate (C₀) into the heart was controlled by changing the volume of the reservoir and the rate of ouabain infusion into the reservoir fixed in front of the heart. The time courses of PIA were measured continuously with different infusion rates. The relationship between C₀ and PIA clearly depended on the infusion rate in isolated perfused rabbit heart. The binding kinetics of ouabain to Na⁺, K⁺-adenosine triphosphatase (ATPase) in the cardiac homogenate showed two kinds of binding sites. The association rate constant (k₁), the dissociation rate constant (k₋₁) and the binding capacity of each site was estimated by the simultaneous fitting method. The occupation curve of the high affinity site corresponded well with the PIA measured in the isolated perfused heart at steady state. These results indicate that ouabain binding to the high affinity site is related to the PIA, and the slow binding process of ouabain to Na⁺, K⁺-ATPase may be one of the principal reasons for the infusion-rate dependence of ouabain PIA.

Keywords — ouabain; positive inotropic action; isolated perfused rabbit heart; Na⁺, K⁺-ATPase; binding; kinetic analysis

Introduction

It is generally considered that drugs administered into the body are carried in the blood to each organ and are transported to the target site, and there they exert their pharmacological effects or toxicity. Sheiner et al. 1) introduced the effect compartment model to explain the difference of the kinetics between the plasma concentration (C₀) and the pharmacological effect when the drug is transported slowly from plasma to the target site or when the pharmacological effect appears slowly after the drug has reached the target site.

The positive inotropic action (PIA) of cardiac glycosides requires much time to reach their maximum effect after intravenous injection. 2,3) The reason they need a long time to exert their PIA is still unknown. Na⁺, K⁺-adenosine triphosphatase (ATPase) was considered to be the pharmacological receptor for PIA. 4,5) Recently, it was reported that two kinds of binding sites were observed in the ouabain binding to Na⁺, K⁺-ATPase in rat, guinea pig and cat. However, the relation among PIA and the binding to Na⁺, K⁺-ATPase and the inhibition of Na⁺, K⁺-ATPase activity seems to be different depend on the species. 6-8)

In our previous study, the PIA and C₀ of ouabain was measured simultaneously by changing the infusion rate in vivo. The relationship between the plasma concentration and the PIA of ouabain depended on the infusion rate, i.e. the higher the infusion rate, the higher the C₀ to exert PIA. This indicates the existence of slow step(s) in the process of PIA. 9)

In this study, we examined the kinetics of the PIA of ouabain using the Langendorff method with isolated perfused rabbit heart to determine whether the infusion-rate dependence of PIA occurs in the isolated heart. An isolated perfused system is suitable for the kinetic analysis of PIA, because we can control the input ouabain concentration (C₀) by changing the volume of the resovoir and the infusion rate into this reservoir. We also determined the binding of ouabain to rabbit cardiac Na⁺, K⁺-ATPase to define the kinetic mechanism of the infusion-rate depen-
dence of PIA observed in the isolated perfused heart as well as that in vivo.

Materials and Methods

Materials — Male, Japanese White rabbits (Nihon Igaku Dobutsu, Tokyo) weighing 1.8—2.5 kg were used. Ouabain was purchased from Sigma Chemical Co. (St. Louis, MO). [3H]-Ouabain (20 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and was at least 98% pure as determined by thin layer chromatography. All other chemicals were commercially available and of analytical grade.

Isolated Perfused Heart — Rabbits were given 1000 U/kg of heparin i.p. and were stunned by a blow on the neck 15 min later. After they were bled from the carotid arteries, the thorax was opened and the heart beating was stopped by cold (4 °C) physiological saline. Then the heart was rapidly removed and transferred to a beaker containing cold Krebs–Henseleit solution of the following composition (in mM): NaCl 118, KCl 4.7, CaCl_2 2.25, MgSO_4 1.18, KH_2PO_4 1.18, NaHCO_3 24.88, glucose 11.1. After removal of adhering tissue a cannula was tied into the stump of the aorta, and the heart was perfused retrogradely at 37 °C according to the Langendorff technique with Krebs–Henseleit solution gassed with 5% CO_2 in oxygen (pH = 7.4). The flow rate was kept at 30 ml/min/heart by means of a roller pump (RP-N2, Furue Science, Tokyo). For the final wet weight of the heart, the mean perfusion rate was 4.55 ± 0.45 ml/g/min (mean ± S.E.M.). The heart was beating spontaneously.

Measurement of Positive Inotropic Action — The left ventricular pressure (P) was measured according to the method reported by Sasaki and Shiraki.[^10] A glass cannula (outer diameter 2 mm; inner diameter 1.5 mm) was inserted via the left atrium into the left ventricle. The glass cannula, the tip of which consisted of a small ball (outer diameter, 4 mm; inner diameter, 3.5 mm) with three holes (diameter, 1.5—2.5 mm), was filled with physiological saline and connected with water-filled polyethylene tubing to a Gould Statham transducer (P231D, Gould Inc., CA). The first derivative of the left ventricular pressure (dP/dt_max) was obtained with an electrical differentiator (RPD-5, Nihon Kohden, Tokyo). We expressed the PIA as the increase of dP/dt_max normalized by the maximum value according to the following equation:

\[
I = \frac{E(t) - E(0)}{E_{max} - E(0)}
\]

(1)

where I is an index of PIA, and E(0), E(t) and E_{max}, are the dP/dt_max at the base line, at time t and at the maximum of PIA, respectively.

Concentration–Response Curve at Steady State: After the cardiac contractile state has become stable (usually within 15 min), ouabain was perfused increasingly from 1 to 1000 nM. The time of perfusion at each concentration was 15 min.

Kinetic Analysis of PIA: The reservoir was fixed in front of the heart to control the input concentration of ouabain. In this study, the reservoir volume was fixed at 20 ml (for the high infusion rate) or 80 ml (for the low infusion rate) and was kept constant during the experiment. Ouabain was infused into the reservoir at the rates of 9.3 nmol/min/heart for the low infusion rate and of 62 nmol/min/heart for the high infusion rate. The time course of PIA was measured continuously using the method described above.

Ouabain Binding to Na⁺, K⁺-ATPase in Heart Homogenates — [3H]Ouabain binding was measured by the rapid filtration technique reported basically by Michael et al.[^11] Each left ventricle was homogenized in the ice-bath by three passes in a Teflon homogenizer for 30 s with a ninefold buffer volume containing 50 mM Tris–HCl, 100 mM NaCl and 2.5 mM MgCl_2. The binding reaction was initiated by adding 50 µl aliquots of 10% homogenate to 950 µl of the incubation mixture containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2.5 mM Na_2ATP, 2.5 mM MgCl_2, 4.7 mM KCl and 20 nM [3H]Ouabain (final homogenate concentration: 0.5%), which had been preincubated for 10 min at 37 °C. At indicated times, aliquots (400 µl) were removed and filtered through a 0.45 µm Milipore filter (Milipore Corp. MA) and then rinsed two times
with 4 ml of ice-cold (4°C) saline. The filters were dissolved in 10 ml of scintillation fluid (toluene, 1 1; Triton X-100, 0.5 l; 1,4-bis 2-(5-phenyloxazol) benzene (0.1 g) and diphenylxazole (4 g), and the radioactivity was measured in a liquid scintillation spectrometer (model 3255; Packard Instruments, Downers Grove, IL). The binding was expressed as the specific binding, by subtraction of the binding in the presence of 500 μM unlabeled ouabain from the total binding. In this experimental condition, the unbound concentration remains unaffected by its reaction with the receptor, because the bound concentration of [3H]ouabain was less than 1% of the total concentration.

The dissociation rate constant (k_−_i) for ouabain were determined by the chase method. An aliquot of homogenates was added to the incubation mixture, incubated for 30 min, then a large excess of unlabeled ouabain was added to the incubation mixture (the final concentration; 500 μM). At indicated times, the radioactivity of ouabain was measured by the method described above.

The concentration-dependency of the ouabain binding was studied by adding unlabeled ouabain up to 9000 nM to the incubation mixture. An aliquot of 10% homogenate was added to this incubation mixture and incubated for 30 min. Then the binding was measured by the same method described above.

Analysis of Ouabain Binding to Na^+, K^+-ATPase — 1) Association: The time course of the binding was fitted to the following equation:

\[
DR = \sum_{i=1}^{2} \frac{R_{m,i}D}{K_i + D} (1 - \exp(-(k_i \cdot D + k_{-i}) \cdot t))
\]

(2)

where \(D, R_{m,i}\) and \(DR\) are the concentration of ouabain, the maximum binding capacity of each site \(i = 1\) and \(2\) and the bound ouabain concentration, respectively, \(k_i\) is the association rate constant, and \(K_i\) is the dissociation constant \((K_i = k_{-i}/k_i)\). The derivation of these equations is shown in the Appendix.

2) Dissociation: The time course of the dissociation curve was fitted to the following equation (see Appendix):

\[
DR = \sum_{i=1}^{2} \frac{R_{m,i}D}{K_i + D} \exp(-k_{-i} \cdot t)
\]

(3)

3) Saturation Kinetics — The data of concentration-dependency was fitted to the following equation (see Appendix):

\[
DR = \sum_{i=1}^{2} \frac{R_{m,i}D}{K_i + D}
\]

(4)

The values of \(k_i, k_{-i}\) and \(R_{m,i}\) were obtained by simultaneous fitting of eqs. 3—5 to the experimental data with a nonlinear least squares method, weighted according to the reciprocal of the concentration. The parameters obtained by simultaneous fitting were as follows (fitted value ± S.D.): \(k_1 = 0.00096 \pm 0.00028\) (min⁻¹/nM), \(k_{-1} = 0.096 + 0.013\) (min⁻¹), \(R_{m,1} = 0.32 + 0.11\) (pmol/ml of 0.5% heart homogenerate), \(k_2 = 0.0039 + 0.0017\) (min⁻¹/nM), \(k_{-2} = 2.1 + 1.1\) (min⁻¹) and \(R_{m,2} = 8.8 + 1.4\) (pmol/ml of 0.5% heart homogenerate). The large difference in the dissociation constant, \(K_i\), between the two sites was mainly due to the difference in the dissociation rate constant \(k_{-i}\) rather than that in the association rate constant, \(k_i\). The capacity of the low affinity site was much larger than that of the high affinity site, however, the contribution of each site to the total binding in the tracer experiment \((D = 20\) nM) was almost the same.

Results

Isolated Perfused Heart

Hearts not subjected to drug treatment \((n = 3)\) were substantially stable over a 3-h perfusion period. Once the contractility and the heart rate had stabilized (usually within 15 min), there was a subsequent loss of less than 15% of the \(dP/dt_{max}\) until the end of the 3-h perfusion period.

Concentration-Response Curve of Ouabain

The concentration–response curve of ouabain in isolated perfused heart is shown in Fig. 1. A small peak of PIA occurred at 1 nM and a large PIA appeared observed between 10—1000 nM.
Fig. 1. Concentration-Response Curve of the PIA of Ouabain in the Isolated Perfused Rabbit Heart

The PIA is described according to Eq. 1. Each point and vertical bar represents the mean and S.E.M. of four experiments. The shaded area represents the calculated range of the occupation of the high-affinity site on Na\(^+\), K\(^+\)-ATPase. This occupation curve was calculated by the following equation: occupation = \( D / (K_i + D) \), where \( D \) and \( K_i \) (\( = k_{-1}/k_0 \)) represent the ouabain concentration and the dissociation constant of ouabain for the high infusion rate. The shaded area was estimated by calculating the error of \( K_i \) considering the propagation of error.\(^{2,13} \)

\[
dK_i = \frac{\partial K_i}{\partial k_{-1}} \cdot dk_{-1} + \frac{\partial K_i}{\partial k_i} \cdot dk_i
\]

thus

\[
K_{1,max} = \frac{k_{-1}}{k_i} + \frac{k_{-1}}{(k_0)^2} \cdot \Delta k_i
\]

\[
= \frac{0.0129}{0.00096} + \frac{0.096}{(0.00096)^2} \times 0.00028
\]

\[= 42.6\]

where \( K_{1,max} \) is the maximum value of the estimated error of \( K_i \).

The first PIA was small but significant compared to the base line (\( p < 0.05 \)). After reaching the maximum PIA, the arrhythmia appeared at 1000 nM. The percent change of \( dP/dt_{\text{max}} \) at 1000 nM was 62.5 ± 13.6% (mean ± S.E.M. \( n = 4 \)). No arrhythmias were present in any of these hearts, except with 1000 nM of ouabain.

**Kinetic Analysis of the PIA of Ouabain**

As shown in Fig. 2, PIA appeared slowly and became constant after 15 min in case of the low infusion rate. This constant value was 89% of the maximum PIA according to the steady state concentration-response curve. In case of the high infusion rate, the PIA appeared rapidly and reached the maximum value within 5 min. Then the arrhythmia followed.

The input ouabain concentration in the perfusate (\( C_i \)) was calculated according to the following equation:

\[
C_i = I/Q \cdot (1 - \exp(-Q/V \cdot t))
\]

where \( I \) (9.3 or 62 nmol/min), \( Q \) (30 ml/min) and \( V \) (20 or 80 ml) represent the infusion rate, the flow rate and the reservoir volume, respect-

Fig. 2. The Time Courses of the PIA of Ouabain in Isolated Perfused Rabbit Heart

The infusion rates of ouabain are 9.3 (△) and 62 (■) nmol/min for the low and high infusion rates, respectively. The PIA was described according to Eq. 1. Each point and vertical bar represents the mean and S.E.M. of three experiments. In the low infusion rate, the final value was fixed at 89% according to the concentration-response curve shown in Fig. 1.

Fig. 3. Effect of the Infusion Rate on the Concentration-Response Profile of the PIA of Ouabain

For the low and high infusion rates, the input ouabain concentration was calculated according to Eq. 2. Then the relationship between the concentration and the response was plotted for each infusion rate. Each point and vertical bar represents the mean and S.E.M. of three of four experiments. ■, steady-state; ●, low infusion rate; △, high infusion rate.

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Fig. 4. The Time Course of the Association of Ouabain with Na⁺, K⁺-ATPase of Rabbit Heart Homogenate

The binding experiment was performed by a rapid filtration technique. The bound concentration was expressed as pm per 0.5% (g/dl) homogenate. Each point and vertical bar represents the mean and S.E.M. of four experiments. The solid line represents the simultaneous fitting curve by Eqs. 3-5.

Fig. 5. The Time Course of the Dissociation of Ouabain from the Na⁺, K⁺-ATPase of Rabbit Heart Homogenate

[3H]Ouabain was preincubated for 30 min as described in Methods. At time zero, an excess amount of unlabeled ouabain was added to the incubation mixture and the subsequent release of [3H]ouabain was measured. The bound concentration was expressed as pm per 0.5% (g/dl) homogenate. Each point and vertical bar represents the mean and S.E.M. of four experiments. The solid line represents the simultaneous fitting curve by Eqs. 3-5.

Fig. 6. Scatchard Plot of [3H]Ouabain Binding to the Na⁺, K⁺-ATPase of Rabbit Heart Homogenate

Each point and vertical bar represents the mean and S.E.M. of four experiments. The bound concentration is expressed as nm per 0.5% (g/dl) heart homogenate. The solid line represents the simultaneous fitting curve by Eqs. 3-5. In this analysis, Eq. 5 was fitted to the data of the concentration dependency and the result was described by the solid line according to the Scatchard analysis.

KCl was several times higher than in the presence of KCl (data not shown). An initial concentration of ATP, 2.5 mM was sufficient to support [3H]ouabain binding. The non-specific binding of ouabain to heart homogenate was below 1%. In this experiment, we fixed the potassium concentration at 4.7 mM, the same as that in the isolated perfused heart experiment. The dissociation process did not follow the simple first order kinetics (Fig. 5). The data of the saturation kinetics were plotted by the Scatchard method which clearly showed the presence of two kinds of binding sites (Fig. 6).

Discussion

The in vivo infusion rate dependence of the PIA of ouabain was shown in our previous study. The first purpose of this study is to examine whether the infusion-rate dependence of the PIA of ouabain is effected appear through the central nervous system or is in the isolated heart. For this, we established the isolated perfused heart system with which we can control the C4 as we did in the in vivo study.

As shown in Fig. 1, the half-maximum PIA occurred at 100 nM and the maximum PIA occurred at 1000 nM. This PIA was well correlated
with the occupation curve which was calculated using the dissociation constant of the high affinity site obtained from the binding experiment. In this study, we used equation 5 to calculate the occupation by assuming that the $C_i$ is equal to the unbound concentration of ouabain at steady-state. This correlation indicates that the PIA of ouabain is related, with the ouabain binding, to the high affinity site of Na⁺, K⁺-ATPase. The half-maximum inhibition concentration of Na⁺, K⁺-ATPase activity by ouabain in the rabbit was reported to be 2000 nM using cardiac membrane Na⁺, K⁺-ATPase and this value was almost the same as the occupation of the low affinity site measured in our study. Our results agreed well with the observation in guinea pig in which the ouabain binding to the high affinity site on the cell membrane is concerned with the PIA in contracting cardiac muscle and the inhibition of Na⁺, K⁺-ATPase activity is related to the binding to the low affinity site.

Koomer et al. reported that the concentration-dependent biphasic PIA of ouabain occurred in the isolated perfused heart of the rat and guinea pig. The first effect appeared at 1000 nM for rats and 6 nM for guinea pigs. The second effect appeared between 1000 nM and 10 mM for rats, and between 10 and 1000 nM for guinea pigs. Our results also showed a biphasic PIA similar to that observed in guinea pigs. However, the first PIA observed at 1 nM was very small compared to the second PIA. Therefore the low concentration effect of ouabain may be due to the release of catecholamines.

In the in vivo condition, the relationship between the plasma concentration and the PIA shifted depending on the infusion rate of ouabain. This suggests that the effect compartment of ouabain does not exist in the plasma compartment. In this study, this infusion-rate dependence of the PIA was also occurred in isolated perfused heart. These findings indicate that the slow onset of PIA occurred not via the central nervous system but in the heart itself. The binding kinetics show the slow association and dissociation processes of ouabain relative to the high affinity site of Na⁺, K⁺-ATPase. The dissociation rate constant for the low affinity site, $k_{-2}$, shows a very rapid equilibration time of binding ($t_{1/2} = 1 n 2/k_{-2} = 0.33$ min). But $k_{-1}$ shows a slow equilibration time ($t_{1/2} = 1 n 2/k_{-1} = 7.2$ min), which is like the equilibration time (15 min) of the PIA in the isolated perfused heart. Therefore, this slow process of ouabain binding to the high affinity site may be the principal cause of the slow appearance of the PIA of ouabain. Recently, it was reported that the cardiac cell membrane has two specific binding sites for ouabain in rats and guinea pigs. Since the characteristics of the [³H]ouabain binding to crude homogenates were qualitatively similar to [³H]ouabain binding to more purified preparations of Na⁺, K⁺-ATPase, we did not attempt to purify the Na⁺, K⁺-ATPase but disturbed the cell membranes minimally by preparing homogenates of the left ventricles. We also found two binding sites in rabbit heart and estimated the association and dissociation rate constants for both sites. In this study, the association and dissociation rate constants and the binding capacity for each binding site were determined using the time courses of the association and dissociation and the concentration dependency of the ouabain binding to Na⁺, K⁺-ATPase, simultaneously. This simultaneous fitting method gave more accurate parameters with the minimal assumptions described in the Appendix. Our results show that the association rate constants for both sites were almost the same, however, the dissociation rate constants differed almost twentyfold. This indicated that the difference in the dissociation rate constant may determine the difference in the affinity of ouabain, which was well correlated qualitatively with the result obtained using rat cultured cells. The kinetic rate constants of ouabain binding to beef brain were measured by Yoda et al. using an enzymatic assay. They suggested that the association occurs as follows: first, the association of the steroid moiety of the cardiac glycoside with the steroid-specific site of the enzyme occurs, and then, the conformational change of the sugar specific site follows, and finally, the association of the sugar moiety with the sugar specific site may occurs. In contrast, the sequence of the dissociation reaction is quite the reverse and the rate-limiting step of the dissociation may be a conformational change of the enzyme.
In conclusion, we observed the infusion-rate dependence of the PIA in isolated perfused rabbit heart, and found that the delayed onset of PIA occurred in the heart itself. A kinetic study of the ouabain binding to Na⁺, K⁺-ATPase showed two kinds of binding sites. The concentration-response curve of PIA for ouabain was well correlated with the occupation curve of Na⁺, K⁺-ATPase calculated from the dissociation constant of the high affinity site. The slow binding process of ouabain to Na⁺, K⁺-ATPase may be one of the principal reasons for the infusion-rate dependency of PIA.

Appendix

Calculation of the Binding Parameters of Two Binding Sites

1) Association Rate Constants \((k_i)\) — We assumed that each binding occurred independently and is shown in the following form:

\[
D + R_i \xrightleftharpoons[k_{-i}]{k_i} DR_i
\]  

(A-1)

The rate of this reaction is given by

\[
\frac{dDR_i}{dt} = k_i \cdot D \cdot R_i - k_{-i} \cdot DR_i
\]  

(A-2)

Assuming that the concentration of the drug, \(D\), remains unaffected by its reaction with the receptor and substituting \(R_i = R_{m,i} - DR_i\), we obtain

\[
\frac{dDR_i}{dt} = k_i \cdot D \cdot R_{m,i} - (k_i \cdot D + k_{-i}) \cdot DR_i
\]  

(A-3)

The rate equation for the receptor binding is derived by integrating Eq. A-3 with the initial condition, namely at \(t = 0\), \(DR_i = 0\)

\[
DR_i = \frac{k_i \cdot D \cdot R_{m,i}}{k_iD + k_{-i}} (1 - \exp(-(k_i \cdot D + k_{-i}) \cdot t))
\]  

(A-4)

We obtained the following equation using the relation; \(K_i = k_{-i}/k_i\):

\[
DR_i = \frac{R_{m,i} \cdot D}{K_i + D} (1 - \exp(-(k_i \cdot D + k_{-i}) \cdot t))
\]  

(A-5)

Thus, the bound concentration of ouabain in both sites can be described as follows:

\[
DR = DR_1 + DR_2
\]  

\[
= \sum_{i=1}^{2} \frac{R_{m,i} \cdot D}{K_i + D} (1 - \exp(-(k_i \cdot D + k_{-i}) \cdot t))
\]  

(A-6)

2) Dissociation Rate Constants \((k_{-i})\) — In measuring the dissociation rate constant, we used the chase method as described in Methods. In the presence of excess amounts of unlabeled ouabain, we can obtain the following equation by neglecting the minuend of Eq. A-2:

\[
\frac{dD^*R}{dt} = \sum_{i=1}^{2} -k_{-i} \cdot D^*R_i
\]  

(A-7)

where \(D^*\) represents the concentration of \([3H]\)ouabain. With the initial condition,

namely \(t = 0\), \(DR_i = \frac{R_{m,i}D^*}{K_i + D^*}\), we obtain the following equation:

\[
D^*R = \sum_{i=1}^{2} \frac{R_{m,i} \cdot D^*}{K_i + D^*} \exp(-k_{-i} \cdot t)
\]  

(A-8)

3) Dissociation Constants \((K_i)\) — We can describe the binding at equilibrium by the following equation from Eq. A-6:

\[
DR_e = \sum_{i=1}^{2} \frac{R_{m,i} \cdot D}{K_i + D}
\]  

(A-9)

where \(D\), \(DR\), \(R_{m,i}\) and \(K_i\) represent the unbound ouabain concentration, the bound ouabain concentration, the maximum binding capacity and the dissociation constant, respectively. Subscript \(i\) represents sites 1 and 2, and subscript \(e\) represents the equilibrium condition. In addition, the unbound ouabain concentration
can be considered equal to the total ouabain concentration, since \( D \) is much larger than \( DR \) (see Methods).

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