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Apparent cooperativity of [^{3}H]ouabain binding to myocytes obtained from guinea-pig heart

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Kinetics of [^{3}H]ouabain binding to intact cardiac cells were examined using myocytes obtained from guinea-pig heart. In intact cells, the use of excess unlabeled ouabain results in an under-estimation of nonspecific binding, presumably due to cytotoxic effects of the unlabeled glycoside; estimation of the specific binding, as that to rapidly releasing sites yields more accurate results. Specific [^{3}H]ouabain binding to myocytes is promoted by an increase in Na^+ influx, indicating that normal intracellular Na^+ concentration is insufficient to fully stimulate glycoside binding. High concentrations of [^{3}H]ouabain seem to increase the apparent affinity of binding sites for the glycoside via increases in intracellular Na^+ concentration resulting from sodium-pump inhibition; hence the binding reaction may be regarded as having a novel type of cooperativity. This cooperativity has kinetics different from those of classical positive cooperativity based on binding-site interactions, and is apparent with toxic concentrations of the glycoside that cause marked increases in intracellular Na^+ concentrations.

Introduction

Specific binding of [^{3}H]ouabain to isolated ($Na^+ + K^+$)-ATPase observed in the presence of Na^+, Mg^{2+} and ATP represents binding of the glycoside to its pharmacological and toxic receptors in heart muscle [1]. Under these conditions, the glycoside preferentially binds to the Na^+-induced conformation of the enzyme which is increased in the presence of Na^+ and reduced by K^+ [2–7]. Accordingly, affinity of ($Na^+ + K^+$)-ATPase for the cardiac glycosides in intact cells is increased by raising intracellular Na^+ and reduced by raising extracellular K^+ concentrations [3,8–10].

In isolated enzyme preparations, no cooperativity in glycoside binding has been observed when the binding was supported by Na^+, Mg^{2+} and ATP [11], i.e., when the glycoside binding occurs via the pathway that represents binding in intact cells [1]. In intact cells, however, inhibition of the sodium pump resulting from glycoside binding to ($Na^+ + K^+$)-ATPase may elevate intracellular Na^+ concentration and thereby promote glycoside binding [12,13]. Therefore, a novel mechanism of the positive cooperativity, based on an indirect interaction of the binding sites via an increase in intracellular Na^+ concentration instead of a direct

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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interaction between binding sites, has been proposed [13,14].

Kinetic analysis of the glycoside binding to the sodium pump in intact tissue has been hampered by problems associated with a high degree of nonspecific binding that may reach 50% of the total binding [12,13,15,16]. Analysis of kinetic parameters for [^{3}H]ouabain binding has been successfully accomplished using isolated myocyte preparations obtained from hearts of adult animals [17,18], or cultured cells derived from hearts in the embryonic stage of development [19–22]. In the latter studies, however, the positive cooperativity of the glycoside binding was not observed. The discrepancy may result from the difficulty in precisely estimating nonspecific glycoside binding in intact tissue preparations, insufficient increase in intracellular Na^+ concentrations under the conditions of the isolated myocyte studies, or differences in ligand conditions chosen for these studies. Studies using intact tissue preparations maintained physiological concentrations of Ca^{2+} and K^+ in the incubation medium, whereas those using myocytes were generally performed in the presence of low concentrations of Ca^{2+} and K^+. In one study with myocytes in which Ca^{2+} and K^+ concentrations were maintained at nearly physiological concentrations [18], the cells were obtained from rat heart. The glycoside may not significantly increase intracellular Na^+ concentration in this species because the bulk of the sodium pump has low affinity for ouabain [18,23].

Therefore, the positive cooperativity of [^{3}H]ouabain binding was examined in the present study in a medium containing physiological concentrations of Ca^{2+} and K^+, using Ca^{2+}-tolerant myocytes obtained from guinea-pig heart. This is the species in which Herzig et al. [13,14] have suggested the presence of positive cooperativity for [^{3}H]ouabain binding.

Materials and Methods

Isolation of myocytes

Myocytes were obtained from guinea-pig heart by the following method. Hearts were perfused via the aorta (Langendorff preparations) for 15 min with Krebs-Henseleit bicarbonate buffer (KHB) solution containing 118 mM NaCl, 27.1 mM $NaHCO_3$, 2.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate and 10 mM dextrose. The solution was saturated with a 95% O_2/5% CO_2 gas mixture yielding a final pH value of 7.4. Following this washing period, hearts were perfused with a modified KHB solution containing 105.1 mM NaCl, 20 mM $NaHCO_3$, 2.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.01 mM $CaCl_2$, 5 mM mannitol, 10 mM taurine, 10 mM dextrose, 5 mM sodium pyruvate, and saturated with the above gas mixture. Langendorff preparations ceased to contract during this perfusion. Subsequently, collagenase (0.52 mg/ml) and hyaluronidase (0.2 mg/ml) were added to the solution which was recirculated for 50 min. Following digestion with collagenase and hyaluronidase, ventricles were minced in a KHB solution containing 1.4 mM $CaCl_2$. Myocytes were disaggregated by gentle agitation with a wide-mouthed pipette, and rod-shaped cells were selected by centrifugation, elutriation and gravity sedimentation. After isolation, myocytes were maintained up to 4 h in a KHB solution containing 1.8 mM $CaCl_2$.

Viability of myocytes was assessed by counting cells with a hemocytometer. Rod-shaped cells were considered to be viable. Cells were sampled for the determination of viability by pipetting 0.3 ml of cell suspension into 2 ml of 10% formalin. Dispersion of cells in formalin resulted in their shape being maintained until samples could be examined.

[^{3}H]Ouabain-binding studies

Myocytes (0.2–0.4 mg protein) were incubated with [^{3}H]ouabain (0.05–3 μM) at 37 °C for 60 min in KHB solution containing 1.8 mM $CaCl_2$ under gentle swirling. Either rapid filtration or a centrifugation method was used to separate bound and free [^{3}H]ouabain. The centrifugation method has advantages of efficient washing of the external surface of the cells during sedimentation through a sucrose layer, which is free from the labeled ligand, and also sampling only a part of a large pellet. The filtration method has the advantage of better time resolution. In the filtration method, the binding reaction was stopped by the addition of an ice-cold solution containing 0.1 mM unlabeled ouabain, 15 mM KCl and 50 mM Tris-HCl

buffer (pH 7.5). the mixture was filtered through a sialized 24 mm fiberglass filter (Boehringer-Mannheim, Indianapolis, IN). Filters and myocytes were washed two times with 5 ml of the above stopping solution. Filters were treated with a tissue solubilizer (Protosol, New England Nuclear, Boston, MA) and counted for [^{3}H]ouabain using a liquid scintillation spectrometer.

In several experiments, bound and unbound [^{3}H]ouabain were separated by centrifugation of myocytes through a sucrose layer. Centrifuge tubes for sampling were prepared by pipetting 4 ml of a solution containing 280 mM sucrose, 20 mM procaine-HCl (added to stabilize the cell membrane) and 0.9 mM $CaCl_2$ into 15 ml polypropylene tubes (Becton-Dickerson tubes from Sergent-Welch, Livonia, MI). Subsequently, 9 ml of a less dense solution containing 99 mM NaCl, 30 mM RbCl, 20 mM procaine · HCl, 10 mM Hepes, 2.0 mM $BaCl_2$, 0.9 mM $CaCl_2$, 0.1 mM ouabain and a trace amount of Patent blue violet dye (pH 7.1 adjusted with 2 M NaOH) was added slowly to form a distinct layer. These 'sampling tubes' were placed in a bath at $-2°C$ and were allowed to stand for at least 1 h before use.

Prior to sampling, myocytes incubated with [^{3}H]ouabain were allowed to settle in incubation tubes to as great an extent as possible within the confines of individual experiments or 3 min, whichever was less. At the time when the [^{3}H]ouabain-binding reaction was to be stopped, 0.5 ml of incubation mixture containing the cells was taken from the bottom of incubation tubes and added to the top of the above described sampling tubes. Nearly homogeneous dispersion of the sample within the less dense solution yielded a final temperature of $0°C$, thereby effectively stopping the [^{3}H]ouabain-binding reaction. Preliminary results indicate that [^{3}H]ouabain binding did not change significantly when the centrifugation was delayed for up to 15 min after samples were added to centrifuge tubes.

Cells were collected by centrifuging the samples for 2 min at $1500 \times g$ in a refrigerated centrifuge maintained at $2°C$. The layer of less dense solution, identified by its blue color and containing free [^{3}H]ouabain, was aspirated leaving approx. 3 ml of a sucrose solution covering the cell pellet. Tubes were immersed in methanol at $-30°C$ for rapid freezing. Cell pellets were collected by cutting the bottom of the frozen tubes, dried overnight at room temperature, wetted with 75 μl of distilled water, digested using tissue solubilizer (Protosol), and the radioactivity of [^{3}H]ouabain was estimated using a liquid scintillation spectrometer. The addition of a small amount of distilled water was necessary to maintain the sucrose in solution after the addition of scintillation fluid.

The time-course of the dissociation of [^{3}H]ouabain from myocytes was examined after a 60 min incubation for [^{3}H]ouabain binding. An aliquot of the loose pellet was centrifuged as above to determine total [^{3}H]ouabain binding at the end of the binding reaction. The remainder of the cell pellet was dispersed in a 100-fold volume of KHB solution containing 0.2 μM unlabeled ouabain. After resuspension, 6.0 ml of well-mixed cell suspension was removed at predetermined time intervals for up to 3 h, and cells were collected by filtering the suspension through glass filters as described above. Supernatant solutions were centrifuged for 2 min at $1500 \times g$ to remove cells. and treated in a manner identical to that used for sampling of cell suspensions to estimate the background [^{3}H]ouabain binding to filters.

In [^{3}H]ouabain-binding studies for the analysis of kinetic parameters, cells were incubated at $37°C$ for 60 min as above. Total [^{3}H]ouabain binding to myocytes was assayed by the above centrifugation method. Nonspecific [^{3}H]ouabain binding was estimated after allowing 60 min for dissociation of [^{3}H]ouabain. Dissociation of [^{3}H]ouabain was initiated by terminating the binding reaction by mixing cells with a 100-fold volume of KHB solution containing 13.8 mM KCl and unlabeled ouabain. The concentration of unlabeled ouabain was equal to that of [^{3}H]ouabain during the incubation, thereby reducing the specific radioactivity of [^{3}H]ouabain to 1%. Dissociation of [^{3}H]ouabain was allowed to proceed for 60 min at $37°C$, and then cells were collected by centrifugation. Specific binding of [^{3}H]ouabain was calculated as the difference in total and nonspecific [^{3}H]ouabain binding (see below for the rationale for estimating nonspecific [^{3}H]ouabain binding in this manner).

Miscellaneous

Protein concentration was determined by the

method of Bradford [24] using Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Richmond, CA). [^{3}H]Ouabain (generally labeled, spec. radioact. 20.0 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Collagenase was obtained from Cooper Biomedical, Malvern, PA., Boehringer-Mannheim, Indianapolis, IN, and Sigma Chemicals, St. Louis, MO. Hyaluronidase and monensin were purchased from Sigma Chemicals. Tissue solubilizer (Protosol) was purchased from New England Nuclear.

Statistical analysis was performed using grouped t-test, paired t-test or analysis of variance with $P < 0.05$ as the criterion for statistical significance.

Results

Estimation of nonspecific [^{3}H]ouabain binding

In the kinetic analysis of [^{3}H]ouabain binding, it is essential to accurately estimate nonspecific binding, because inaccurate subtraction of 'nonspecific binding' causes the curve on Scatchard plots to bend upwards (under-estimation of nonspecific binding) or downwards (over-estimation of nonspecific binding). A popular method of estimating the nonspecific binding is to perform binding studies in the presence of an excess unlabeled ouabain which displaces [^{3}H]ouabain from specific binding sites. This method is apparently satisfactory in isolated ($Na^+ + K^+$)-ATPase preparations. In viable myocytes, however, toxicity of high concentrations of unlabeled ouabain may alter characteristics of the myocytes. Therefore, stability of guinea-pig myocytes in a solution containing 1.8 mM $CaCl_2$ and either a low (1 μM) or high (1 mM) concentration of ouabain was examined.

Incubation of myocytes in KHB solution at 37°C for 60 min resulted in a decrease in the percentage of cells retaining a rod shape from 83.5 ± 3.5 to 76.3 ± 2.9%. Preparations incubated with 1 μM ouabain, however, showed no loss of viable (rod-shaped) cells. Presence of 1 mM ouabain caused a marked loss of viable cells, and less than 5% of cells were rod-shaped after a 60 min incubation. Microscopic examination of these cells prior to fixing in formalin showed that the few cells still retaining a rod shape were contracting spontaneously. Moreover, the incubation media became yellow, indicating that cell lysis was occurring (data not shown). These changes in cellular characteristics may affect nonspecific binding of [^{3}H]ouabain causing it to be under- or overestimated. Therefore, it was necessary to examine whether nonspecific [^{3}H]ouabain binding can be accurately estimated in these preparations.

Nonspecific [^{3}H]ouabain binding may be estimated in guinea-pig heart preparations as bound [^{3}H]ouabain remaining after the dissociation of the glycoside from a 'rapid' component because [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase is released from the enzyme with a half-life of several minutes at 37°C in this species [25,26]. Therefore, kinetics of the dissociation of [^{3}H]ouabain from myocytes were examined. Dissociation of [^{3}H]ouabain, observed after a 60 min incubation of myocytes with 0.2 μM [^{3}H]ouabain, occurred in two phases (Fig. 1). [^{3}H]Ouabain bound to the rapidly dissociating site accounted for 84.6 ± 1.1% of bound drug (mean ± S.E. of six experiments).

A semilogarithmic plot of these data produced an upward concave curve (Fig. 2). The portion of the curve for the time points greater than 60 min

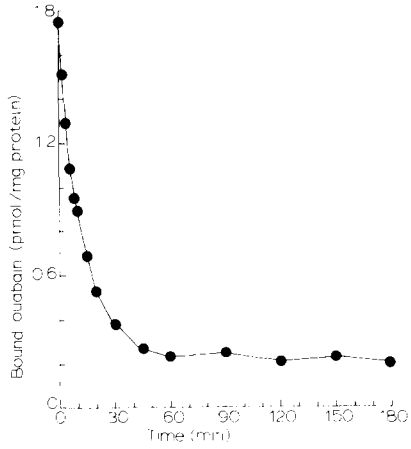

Fig. 1. Time-course of the dissociation of [^{3}H]ouabain from myocytes. Myocytes from guinea-pig heart were incubated at 37°C for 60 min in the presence of 0.2 μM ouabain. Cells were collected by gravity sedimentation and resuspended in a 100-fold volume of KHB solution at 37°C containing 0.2 μM unlabeled ouabain. The myocyte suspension was sampled by the rapid filtration method at the indicated times following the start of dissociation. Typical results from several experiments.

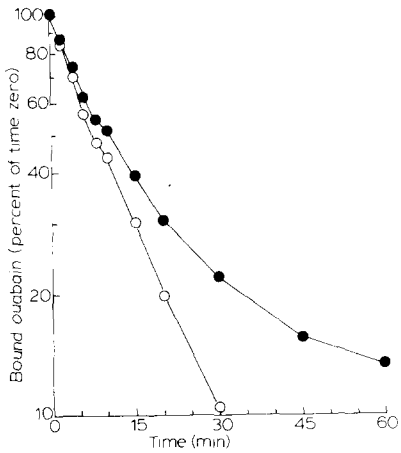

Fig. 2. Semilogarithmic plots for the time-course of [^{3}H]ouabain release from myocytes. The time-course of dissociation was determined as in Fig. 1. Amounts of [^{3}H]ouabain remaining on myocytes were expressed as percentage of bound [^{3}H]ouabain at time zero and plotted on a logarithmic scale. The value at time zero was 1.87 ± 0.10 pmol per mg protein ($n = 6$). ●, total [^{3}H]ouabain bound at indicated time; ○, rapidly dissociating component of bound [^{3}H]ouabain.

appeared to become a straight line roughly parallel to the X-axis. Estimates of [^{3}H]ouabain bound to myocytes after 60 min or longer of dissociation were not sufficiently precise to allow the fitting of a straight line and its extrapolation to zero time. Because the curve after 60 min appeared to be flat, the 60 min value was used as an estimate of the 'residual' binding. Subtraction of this value from the amount of bound [^{3}H]ouabain remaining at earlier time points produced a linear semilogarithmic plot (Fig. 2, open symbols). This single-exponential process has a dissociation rate constant (k_{-1} value) of 0.077 ± 0.01 per min corresponding to a half-life of 9.0 ± 0.7 min at 37°C (mean $\pm$ S.E. of six experiments) which is in good agreement with the dissociation rate of [^{3}H]ouabain from ($Na^+ + K^+$)-ATPase isolated from guinea-pig heart [25,26].

This method of estimating nonspecific binding was compared with values obtained by incubating myocytes and [^{3}H]ouabain in the presence of 1 mM unlabeled ouabain. Two methods for separating bound and unbound [^{3}H]ouabain, namely filtration and centrifugation methods, were also compared. Estimates of the total [^{3}H]ouabain binding were not significantly different when cells

TABLE I

[^{3}H]OUABAIN BINDING TO MYOCYTES

Myocytes from guinea-pig heart were incubated in the presence of 0.1 μM ouabain for 60 min. Total [^{3}H]ouabain bound was determined after separating bound and free ligand by the indicated sampling method.

Sampling method	N	Total	[^{3}H]ouabain bound	
			ouabain-insensitive [a] (pmol per mg) protein)	slowly dissociating [b] (pmol per mg protein)
Filtration	8	1.11 ± 0.08	0.058 ± 0.08	0.212 ± 0.024 [d]
Centrifugation	8	1.12 ± 0.05	0.026 ± 0.002 [c]	0.182 ± 0.009 [d]

[a] Nonspecific binding estimated by including 1 mM unlabeled ouabain in the incubation medium.

[b] Nonspecific binding estimated by allowing [^{3}H]ouabain to dissociate from the myocytes for 60 min at 37°C after termination of the binding reaction.

[c] Significantly different ($P < 0.05$) from the corresponding value obtained with filtration method. Statistical analyses were performed with Student's t-test.

[d] Significantly different ($P < 0.05$) from nonspecific binding estimated using 1 mM unlabeled ouabain.

were sampled by either filtration or centrifugation (Table I). The estimates for [^{3}H]ouabain retained by cells after a 60 min dissociation were also not affected by the method of sampling. Nonspecific binding estimated as [^{3}H]ouabain bound in the presence of 1 mM unlabeled ouabain was significantly higher when the rapid-filtration method was used to separate unbound ouabain. More importantly, the use of 1 mM ouabain to estimate nonspecific binding of [^{3}H]ouabain to myocytes resulted in values which were significantly lower than those obtained with the 'dissociation' method (Table I), regardless of the method used to collect samples. Because of the extreme morphological change, including lysis of the cells that occurred during incubation of myocytes in the presence of 1 mM ouabain and 1.8 mM $CaCl_2$, the dissociation method was considered to be more accurate for estimation of nonspecific binding.

Specific [^{3}H]ouabain binding

Time-courses for [^{3}H]ouabain binding to rapidly and slowly releasing sites were examined to determine the optimum incubation time for

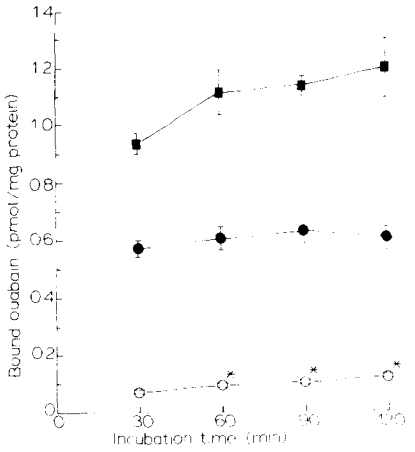

Fig. 3. Binding of [^{3}H]ouabain to rapidly and slowly dissociating sites. Myocytes from guinea-pig heart were incubated in the presence of 50 nM [^{3}H]ouabain for the indicated time in the absence or presence of 2 μM monensin. Bound [^{3}H]ouabain was estimated after the indicated incubation time by collecting myocytes with the rapid-filtration method for determination of total bound [^{3}H]ouabain or estimated after a 60 min incubation of resuspended myocytes to allow bound [^{3}H]ouabain to dissociate from the rapidly dissociating sites. ●, binding to rapidly dissociating sites in the absence of monensin (the difference between total bound [^{3}H]ouabain and that remaining after a 60 min incubation for dissociation from rapidly dissociating sites); ■, binding to rapidly dissociating sites in the presence of 2 μM monensin; ○, [^{3}H]ouabain remaining bound after 60 min of dissociation (binding and uptake to slowly releasing sites; nonspecific binding). Each point represents the mean of five experiments. Vertical lines indicate S.E.; *, significant increase in [^{3}H]ouabain binding during the 30 min incubation.

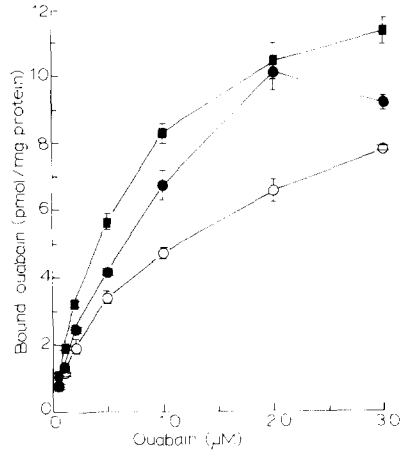

Fig. 4. Specific [^{3}H]ouabain binding to myocytes from guinea-pig heart in the absence (○) and presence (●) of 2 μM monensin, or in the absence of Ca^{2+} (■). Myocytes were incubated in KHB solution containing 1.8 mM $CaCl_2$ or 0.25 mM EGTA at 37 °C for 60 min with the indicated concentration of [^{3}H]ouabain. Specific binding was calculated as the difference between total binding and [^{3}H]ouabain remaining bound after 60 min of dissociation.

equilibrium binding studies. Myocytes were incubated in KHB solution containing 0.05 μM [^{3}H]ouabain for 30 to 120 min. [^{3}H]Ouabain associated with the rapidly releasing component increased with time, reaching an apparent plateau between 30 and 60 min (Fig. 3). Due to the scale, it may not be apparent from Fig. 3; however, nonspecific [^{3}H]ouabain binding observed as above using the dissociation method increased significantly with each 30 min period during the 120 min incubation for the binding reaction. These results indicate that a 60 min incubation is adequate for [^{3}H]ouabain binding studies with guinea-pig myocytes.

Specific [^{3}H]ouabain binding to myocytes observed after a 60 min incubation increased with increasing [^{3}H]ouabain concentrations between 0.05 and 3 μM (Fig. 4). The presence of 2 μM monensin, the Na^+ ionophore which increases Na^+ influx without causing toxicity in guinea-pig atrial muscle preparations [8], caused a significant in-

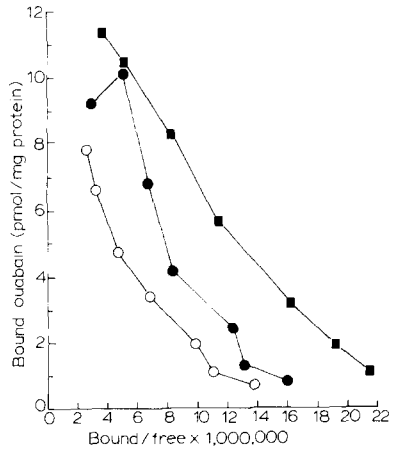

Fig. 5. Scatchard plots for specific [^{3}H]ouabain binding to myocytes from guinea-pig heart. Myocytes were incubated in a modified KHB solution containing concentrations of [^{3}H]ouabain ranging between 50 nM and 3 μM for 60 min at 37 °C. The incubation medium contained: ○, 1.8 mM $CaCl_2$ ($N = 4$); ●, 1.8 mM $CaCl_2$ and 2 μM monensin ($N = 4$); or ■, 0.25 mM EGTA and no added $CaCl_2$ ($N = 3$).

crease in the specific [^{3}H]ouabain binding. Enhancement of the specific [^{3}H]ouabain binding is not the result of changes in the time-course of glycoside binding, because there were no significant differences in the time-courses for [^{3}H]ouabain binding observed in the presence or absence of 2 μM monensin (Fig. 3).

Scatchard analysis of the data shown in Fig. 4 indicates that [^{3}H]ouabain binding observed in the absence of monensin is represented by an upward concave curve (Fig. 5). The presence of 2 μM monensin caused a shift in the curve on Scatchard plots reflecting increased [^{3}H]ouabain binding; however, monensin did not appear to reduce the degree of nonlinearity of the curve.

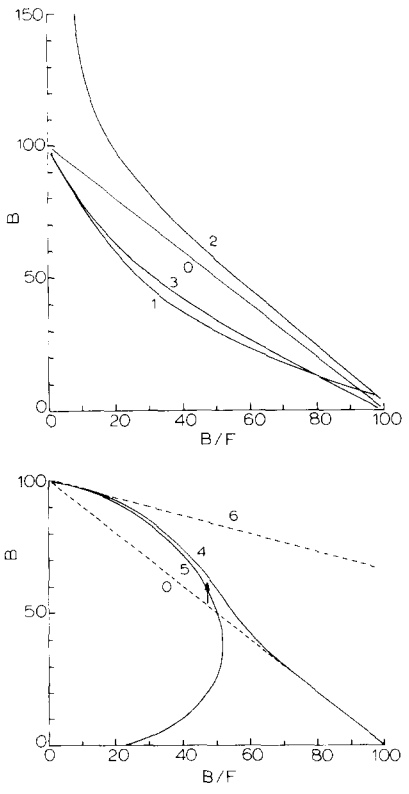

Fig. 6. Theoretical basis for curved Scatchard plots. Curved Scatchard plots such as shown in Fig. 5 may be caused by the following mechanisms: (1) the presence of multiple binding sites; (2) underestimation of nonspecific binding; (3) negative cooperativity; or (4) 'positive cooperativity' caused by increases in intracellular Na^+ concentration resulting from sodium-pump inhibition. The curve representing linear Scatchard plots (0) and positive cooperativity among binding sites (5) are also shown. Curves represent plots for B vs. B/F for following equations:

$$B = (B_t K_1 F)/(1 + K_1 F) \quad (0)$$

$$B = (B_1 K_1 F)/(1 + K_1 F) + (B_2 K_2 F)/(1 + K_2 F) \quad (1)$$

$$B = (B_t K_1 F)/(1 + K_1 F) + C_1 F \quad (2)$$

$$B = 0.5 B_t (K_1 F + 2 K_1 K_2 F^2)/(1 + K_1 F + K_1 K_2 F^2) \quad (3)$$

See explanation below. (4)

$$B = 0.5 B_t (K_1 F + 2 K_1 K_2 F^2)/(1 + K_1 F + K_1 K_2 F^2) \quad (5)$$

$$B = (B_t K_1 F)/(1 + K_1 F) \quad (6)$$

In these equations, B is the specific binding; B_t, maximal binding which is arbitrary set to 100; K_1 and K_2, association constants as defined by Klotz and Hunston [35]; F, free drug concentration; C_1F, residual (unsubtracted) nonspecific binding which is proportional to free drug concentration. In Eqn. 0, $K_1 = 1$. In Eqn. 1, B_1 and B_2 are 30 and 70, respectively, and $K_1 = 3$, $K_2 = 0.3$. In Eqn. 2, $C = 3$. In Eqn. 3, $K_1 = 2$ and $K_2 = 0.2$. In Eqn. 5, $K_1 = 0.5$ and $K_2 = 2$. In Eqn. 6, the value of K_1 is 3.

Curve (4) represents 'positive cooperativity' based on increases in intracellular Na^+ concentrations. The curve follows Eqn. 0 for lower affinity when fractional occupancy of the receptor (B/B_t) is small, and Eqn. 6 for higher affinity, when significant sodium-pump inhibition corresponding to high levels of receptor occupancy raises intracellular Na^+ concentrations. The arrow indicates displacement of the point due to Na^+-induced increase in glycoside binding.

When Ca^{2+} was removed from the incubation medium, there was a greater increase in the specific [^{3}H]ouabain binding resulting in a greater shift in the curve on Scatchard plots. In addition, the plot became more linear when Ca^{2+} was removed from the incubation medium.

Discussion

Present results indicate that Scatchard plots for [^{3}H]ouabain binding to ventricular myocytes of guinea-pig heart observed in the presence of physiological concentrations of Ca^{2+} and K^+ are represented by an upward concave curve. Possible causes for such curved Scatchard plots are: (1) the presence of multiple binding sites with different affinities; (2) an underestimation of nonspecific binding; (3) negative cooperativity among binding sites; or (4) changes in intracellular Na^+ concentration caused at high concentrations of ouabain [13,14]. Among these possibilities, the presence of multiple binding sites with different affinities does not appear tenable, because glyc-

oside binding to homogenates obtained from myocytes shows only one type of binding site in guinea-pig heart [27], and because removal of Ca^{2+} from the incubation medium made the curve to be more linear by increasing glycoside binding especially at low and intermediate concentrations (Fig. 5).

An accurate estimation of nonspecific binding with intact cells is difficult. Conventional means of using high concentrations of unlabeled ouabain to competitively inhibit the specific binding yields apparently satisfactory results with isolated enzyme preparations [2] but does not seem to provide an accurate estimate of nonspecific binding in myocytes. This is because competitive inhibition of specific [^{3}H]ouabain binding by unlabeled glycoside requires concentrations that cause a complete inhibition of the sodium pump, and because antagonists for the cardiac glycosides are presently unavailable. Marked changes in characteristics of the cells resulting from cytotoxicity of high concentrations of the glycoside, including cell lysis, suggest that both specific and nonspecific binding could be altered.

Bound [^{3}H]ouabain estimated before and after partial dissociation at 0 °C has been used to estimate specific and nonspecific binding to guinea-pig skeletal and heart muscle [16]. This method appears to provide a better estimate of nonspecific binding, because it circumvents the exposure of myocytes to toxic concentrations of the glycoside. After a 60 min dissociation reaction at 37 °C used in the present study (6.7-times the half-life of ouabain bound to rapidly dissociating sites), more than 99% of the glycoside should have been released from this site. Dissociation reaction was performed at 37 °C in the present study to facilitate dissociation of [^{3}H]ouabain specifically bound to $(Na^+ + K^+)$-ATPase. Similar experiments may be difficult to perform at 0 °C, because the half-life for the release of bound ouabain from $(Na^+ + K^+)$-ATPase obtained from guinea-pig heart is more than 4 h at 0 °C (data not shown). Similarly, the specific [^{3}H]ouabain binding may not be estimated in canine or feline myocytes using this method, because of the slow release of [^{3}H]ouabain from $(Na^+ + K^+)$-ATPase in these glycoside-sensitive species [25]. The present results are at variance with those by Kjeldsen et al. [16]. These investigators reported that slowly released component probably represents the specific [^{3}H]ouabain binding, whereas nonspecific binding is represented by the faster component. The discrepancy is probably due to the fact that Kjeldsen et al. [16] observed the dissociation reaction at 0 °C. Because the specific component has a high temperature coefficient [25] compared to nonspecific component, it may be anticipated that the dissociation of [^{3}H]ouabain from nonspecific sites proceeds more rapidly than dissociation from specific binding sites at 0 °C, whereas this relationship is reversed at 37 °C.

The true identity of the rapidly dissociating binding sites is not established; however, the dissociation is represented by a single-exponential process and it seems to be associated with $(Na^+ + K^+)$-ATPase because there is a good agreement between the half-lives for [^{3}H]ouabain bound to rapidly dissociating sites in myocytes (present study) and that bound to isolated $(Na^+ + K^+)$-ATPase obtained from guinea-pig heart [25,26]. Moreover, the bulk of the glycoside-binding sites observed with isolated myocytes has been shown to correspond to the glycoside-binding sites on $(Na^+ + K^+)$-ATPase, and occupancy of these sites by [^{3}H]ouabain caused corresponding inhibition of the sodium pump [27]. Because specific binding sites other than $(Na^+ + K^+)$-ATPase do not appear to exist in these cells [27], it is unlikely that the slowly dissociating component also represents the specific [^{3}H]ouabain binding.

[^{3}H]Ouabain which is taken up into nonspecific sites should be released from myocytes when cells are incubated with a solution that does not contain [^{3}H]ouabain. Therefore, the value obtained after a 60 min dissociation reaction is an underestimation of [^{3}H]ouabain taken up into this component at the end of the binding reaction. The curve on semilogarithmic plots after 60 min of dissociation, however, was nearly parallel to the X-axis, indicating that release of [^{3}H]ouabain from nonspecific sites is relatively slow and the underestimation, if any, is relatively small. The removal of Ca^{2+} from the incubation medium caused the Scatchard plots to be nearly linear, indicating that the cause of the curved Scatchard plots is not the underestimation of nonspecific binding. It should also be pointed out that the value of nonspecific

binding estimated using the dissociation method was significantly greater than that estimated using a high concentration of unlabeled ouabain (Table I), suggesting that if there were a systematic error in the estimation of nonspecific binding, it is probably an overestimation instead of an underestimation.

Negative cooperativity among the binding sites should yield Scatchard plots which are represented by an upward concave curve (Fig. 6). It has been reported that the glycoside binding observed in the presence of Mg^{2+} and P_i shows characteristics of the negative cooperativity when K^+ was added to the medium and affinity of the enzyme for the glycoside was markedly reduced [28]. This possibility cannot be ruled out; however, no apparent negative cooperativity among glycoside-binding sites on $(Na^+ + K^+)$-ATPase has been reported in isolated enzyme preparations when binding assay was performed under the 'high affinity condition' [11]. Moreover, no explanation would appear readily available for the present finding that the sodium ionophore, monensin, or the removal of Ca^{2+} from the incubation medium decreases curvature of the Scatchard plots if the curvature is caused by the negative cooperativity. Therefore, cooperativity in cardiac glycoside binding via changes in intracellular Na^+ concentration and concomitant changes in receptor availability, as suggested from experiments using intact tissue [13,14], is the most plausible explanation. According to this hypothesis, intracellular Na^+ concentration in quiescent myocardial cells is insufficient to fully stimulate glycoside binding because only a small fraction of the $(Na^+ + K^+)$-ATPase is in a binding conformation. Ouabain binding observed in the presence of 1–10 mM Na^+ is relatively insensitive to the Na^+ concentration [29]. Therefore, when intracellular Na^+ concentration is in this range, affinity of the enzyme for [^{3}H]ouabain would be independent of the intracellular Na^+ concentration and a linear Scatchard plots (Fig. 6B, curve 0) can be anticipated. In the presence of high concentrations of [^{3}H]ouabain, however, intracellular Na^+ concentration increases. As intracellular Na^+ concentration increases above 15 mM, ouabain binding is stimulated [29], probably because a higher fraction of the $(Na^+ + K^+)$-ATPase takes a binding conformation. This would move the point on Scatchard plots upward (Fig. 6B, arrow) until the intracellular Na^+ concentration becomes sufficiently high to maximally stimulate the glycoside binding at high concentrations of [^{3}H]ouabain (Fig. 6B, curve 6).

The above hypothesis is supported by the finding that 2 μM monensin, which increases Na^+ influx in cardiac muscle [8], increased [^{3}H]ouabain binding, indicating that the intracellular Na^+ concentration in these myocytes is apparently insufficient to maximally stimulate glycoside binding. This is also consistent with the estimated intracellular Na^+ concentration which is less than 10 mM in quiescent cardiac muscle [30,31]. This concentration of Na^+ is apparently insufficient to fully stimulate glycoside binding [4]. Therefore, an increase in intracellular Na^+ concentration is expected to increase [^{3}H]ouabain binding. This concentration of monensin, however, failed to change the shape of Scatchard plots for [^{3}H]ouabain binding from an upward concave curve to a straight line. This finding is not inconsistent with the above hypothesis, because full stimulation of glycoside binding to $(Na^+ + K^+)$-ATPase is expected to require a large increase in intracellular Na^+ concentration, which may not be achieved in viable cells without causing overt toxicity. The concentration of monensin used in the present experiments is not toxic to intact muscle [8], and failed to cause either changes in the shape or increases in spontaneous activity of isolated myocytes. Moreover, 2 μM monensin does not induce maximal activation of ouabain-sensitive $^{86}Rb^+$ uptake, indicating that intracellular Na^+ concentration does not reach saturating level for the sodium pump with this concentration of monensin [32].

Elimination of Ca^{2+} from the incubation medium caused a marked increase in [^{3}H]ouabain binding as reported earlier [33], and changed the shape of the Scatchard plots for [^{3}H]ouabain binding to nearly a straight line. It seems therefore that the Scatchard plots observed in the absence of Ca^{2+} represents true or maximal affinity of $(Na^+ + K^+)$-ATPase for the glycoside, whereas those observed in the presence of Ca^{2+} are curved because of reduced binding at low concentrations of ouabain. Because high concentrations of [^{3}H]ouabain increase apparent affinity of binding

sites for the glycoside via inhibition of the sodium pump and ensuing increases in the intracellular Na^+ concentration, [^{3}H]ouabain binding may be regarded to exhibit 'positive cooperativity'. The shape of the binding curve representing this type of cooperativity, however, is different from that of the curve representing true positive cooperativity (Fig. 6).

In the absence of Ca^{2+}, Scatchard plots for [^{3}H]ouabain binding to isolated myocytes were linear. The mechanism by which elimination of Ca^{2+} markedly increases the glycoside binding is unknown. Mansier and Lelievre [33] claimed that exposure of intact cells to a Ca^{2+}-free medium causes the sodium pump to assume an 'affinity mode', whereas Hohl et al. [34] reported that elimination of Ca^{2+} from incubation media markedly increases intracellular Na^+ concentration. If an increase in intracellular Na^+ concentration caused by the removal of Ca^{2+} is sufficiently high, it would increase affinity of the sodium pump for the cardiac glycoside without causing toxicity which may result from Ca^{2+} overload. Regardless of the cause, however, the above finding would appear to explain the discrepancy between results reported by Herzig et al. [13,14] demonstrating the novel type of cooperativity for [^{3}H]ouabain binding to intact tissue and those reported earlier by several investigators demonstrating linear Scatchard plots for the glycoside binding to single cells. Most single-cell studies [17,19,21,22] were performed in the absence or with a low concentration of Ca^{2+} in the incubation medium.

In summary, [^{3}H]ouabain binding to myocytes isolated from guinea-pig heart is represented by nonlinear Scatchard plots. The curved Scatchard plots result from reduced glycoside binding at low concentrations of [^{3}H]ouabain due to low intracellular Na^+ concentrations in viable myocytes. As the concentrations of [^{3}H]ouabain is increased, the intracellular Na^+ concentration increases, thereby increasing glycoside binding and displacing the Scatchard plots upwards. Because glycoside binding observed at high concentration of [^{3}H]ouabain is increased above the 'ideal line' [35], [^{3}H]ouabain may be regarded to have positive cooperativity. This novel type of 'positive cooperativity', however, does not appear to operate at pharmacological concentrations of the glycoside, because it becomes significant only when the occupancy of the glycoside-binding sites and the degree of sodium-pump inhibition are substantial.

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