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BINDING PROPERTIES AND BIOLOGICAL EFFECTS OF OXIDIZED-OUABAIN ON CULTURED NEONATAL-RAT CARDIAC MYOCYTES

IMPLICATIONS ON THE MECHANISM OF ACTION OF THE DIGITALIS-GLYCOSIDES

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Abstract—Mild oxidation of ouabain with $NaIO_4$, causes the cleavage of the bond between C2′ and C3′ of the rhamnose ring, leaving the steroid moiety intact. The oxidized ouabain (ox-ouabain) was examined on spontaneously contracting cultured rat-cardiac myocytes. Two classes of binding sites, with high and low affinities, were detected for both ox-ouabain and unmodified oubain. The dissociation constants (K_D) were found to be similar for both compounds, but the rate constants of association (k_a) and dissociation (k_d) of the low affinity sites were higher for ox-ouabain as compared with ouabain. Displacement experiments showed that ox-ouabain and ouabain bind to the same sites. The effects of ox-ouabain and ouabain on the activity of Na^+, K^+-ATPase were determined in microsomal preparations. Similar dose–response curves for the inhibition of the enzyme activity were determined for both drugs. Inhibition was observed only at concentrations above 10^{-6} M. The biological effects of the drugs were examined by their capacity to induce positive inotropic or toxic effects. Concentrations of ox-ouabain which induced positive inotropic effects (increase in amplitude of systolic cell motion), ranged from 5×10^{-8} M to 5×10^{-6} M, as compared with 10^{-7} M to 5×10^{-7} M with ouabain. "Toxic" effects (decrease in the amplitude of systolic motion, increased beating frequencies and elevation in the position of maximal relaxation) was observed only with 10^{-5} M ox-ouabain as compared with 10^{-6} M ouabain. The mechanism of the inotropic action of ox-ouabain at the lower concentration range was investigated by measuring the effect of the drugs on $^{86}Rb^+$ (analogue of K^+) influx. Dose–response curves of effects of ouabain and ox-ouabain on $^{86}Rb^+$ influx were bi-phasic. At low concentrations stimulation was observed, whereas at high concentrations $^{86}Rb^+$ influx was inhibited. Ox-ouabain stimulated $^{86}Rb^+$ influx by lower concentrations and to a greater extent than ouabain. A part of $^{86}Rb^+$ influx into cardiac myocytes is mediated by the $K^+/Na^+/Cl^-$ cotransporter, which can be inhibited by loop diuretic drugs such as bumetanide. We have previously shown that ouabain, at low concentrations, stimulates the activity of the cotransporter. It is shown in the present work that ox-ouabain stimulates the activity of the cotransporter by lower concentrations and to a greater extent than ouabain. The inotropic effects at the lower concentration range of ox-ouabain, as compared to ouabain, seem to be caused by the greater stimulation of the cotransporter, and not by the inhibiton of the Na^+, K^+-ATPase activity, which was found to be similar for both drugs. The lower toxicity at the higher concentration range seems to be linked with a lower steady-state level of $[Ca^{2+}]_i$ and a higher rate-constant of dissociation of the low affinity site.

Digitalis glycoside drugs are used in medicine to augment cardiac contractility, but the mechanism of their positive inotropic action on cardiac cells is still controversial [1]. It is accepted that binding of the glycosides to their receptors on plasma membrane Na^+, K^+-ATPase is the first step in their biological effect [2], but several aspects in the pathway, which leads to the increase in the intracellular Ca^{2+}-transients during systole [3, 4] and to the increase of contractile force, are not completely understood. It is well accepted that in chick cardiac myocytes, and at the higher concentration range in mammalian cardiac myocytes, binding of the cardiac glycosides to the receptor leads to inhibition of Na^+, K^+-ATPase. The resulting elevated concentration of intracellular Na^+ leads, through the Na^+/Ca^{2+} exchanger, to an increase in $[Ca^{2+}]_i$,¶ and consequently to positive inotropic or toxic effects according to the concentration of the glycosides [5].

Whereas in chick cardiac myocytes only one class of receptors for ouabain was detected [6, 7], in cardiac preparations from rat and guinea pig, as well as from human, two classes of binding sites for ouabain,

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¶ Abbreviations: Ox-ouabain, oxidized ouabain; $[Ca^{2+}]_i$, concentration of free cytosolic calcium ions; $[Na^+]_i$, concentration of intracellular Na^+; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ASM, amplitude of systolic cell motion.

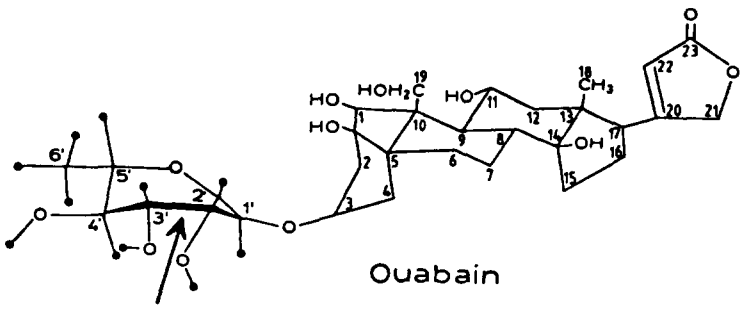

Fig. 1. The chemical structures of ouabain and ox-ouabain. The arrow indicates the bond cleaved by the mild oxidation of ouabain, as determined by ^{1}H NMR spectroscopy [17].

of a high and of a low affinity, were detected [8–10]. Binding of [^{3}H]ouabain to the high affinity sites occurred at concentrations which induced a positive inotropic effect, but K^+ influx was not inhibited, it was rather stimulated [11–13]. Inhibition of K^+ influx and the inotropic or toxic effects, at high concentrations of the cardiac glycosides, were associated with binding to the low affinity sites [14]. These results could not be accommodated with the suggested mechanism of action [15]. Results obtained recently by our group led to a new concept on the mechanism of action of the digitalis drugs at the lower concentration range [13, 16]. The stimulation of $^{86}Rb^+$ influx (an analogue of K^+) by ouabain was found to be mediated by stimulation of the activity of the $Na^+/K^+/Cl^-$ cotransporter, which is sensitive to loop diuretics such as bumetanide and furosamide. It is suggested that such stimulation would increase intracellular Na^+ concentration, and via the Na^+/Ca^{2+} exchanger, increase $[Ca^{2+}]_i$.

In the present work, we proceed to examine the mechanism of action of digitalis glycosides on a model of spontaneously contracting cultures of neonatal-rat cardiac myocytes. We compare the binding properties and physiological effects of ouabain to that of a new derivative obtained by a mild oxidation of ouabain: ox-ouabain. It is shown that ox-ouabain and ouabain display similar dose–response curves of inhibition of Na^+, K^+-ATPase and similar K_D values for ligand binding. On the other hand, the inotropic effect and the stimulation of the butmetanide-sensitive $^{86}Rb^+$ influx were induced by ox-ouabain at a lower concentration range.

MATERIALS AND METHODS

Chemical modification of ouabain. Ouabain was oxidized by the addition of one equivalent of $NaIO_4$ and two equivalents of KH_2PO_4 followed by incubation for 60 min at room temperature. The oxidized ouabain (ox-ouabain) was purified by preparative TLC. The chemical structure of ox-ouabain was identified using ^{1}H NMR spectroscopy. It was found that only the bond between C2′ and C3′ of the rhamnose was cleaved, while the steroid nucleus remained unaffected (Fig. 1) [17]. Tritiated ox-ouabain was similarly obtained by oxidizing [^{3}H]ouabain (21 Ci/mmol).

Preparation of tissue culture. Myocardial cells were isolated from ventricular fragments of hearts from 1-day-old 'Sabra' rats by serial trypsinizations as described previously [18], and suspended in Ham F10 medium containing 20% serum and antibiotics. The cell suspensions were enriched with myocytes by preplating in petri dishes for 1 hr; during this time the fibroblasts became attached to the petri dish. The suspension of myocytes was collected from the plates and diluted with the same medium to 5×10^5 cells/mL. For the measurements of cell contractility, cells were plated on circular glass coverslips (25 mm), and for the measurement of $[Ca^{2+}]_i$ the cells were plated on rectangular glass coverslips (13 × 30 mm). Both types of coverslips were placed inside petri dishes. For the measurement of $^{86}Rb^+$ influx and binding experiments, cells were plated in 25-mm tissue culture petri dishes. The cells were maintained in humidified 5% CO_2–95% air atmosphere at 37° for 3 or 4 days before performing the experiments. At the time of the experiment, the cells were in confluent monolayers and exhibited spontaneous contractions.

Binding of [^{3}H]*ouabain and of* [^{3}H]*ox-ouabain to cultured cardiac myocytes.* Binding of the tritiated compound to intact cultured rat myocytes was done essentially by the method described by Kim *et al.* [19]. Monolayers of myocytes were washed twice with solution I containing (mM): HEPES, 4 (pH 7.4); $CaCl_2$, 0.05; NaCl, 137; $MgCl_2$, 0.5; and glucose, 5, and incubated in the same solution for 5 min. Then, [^{3}H]ouabain or [^{3}H]ox-ouabain, at the indicated concentration, were added for various time intervals. Binding was terminated by washing the monolayers four times with 5 mL portions of ice-cold solution I. NaOH (0.1 M, 0.6 mL) was then added to each plate, and the cells were scraped by a rubber policeman and incubated for 1 hr at 37°. Aliquots were taken for determination of radioactivity using a liquid scintillation spectrometer, and for protein determination by the method of Lowry *et al.* [20].

To measure the dissociation rate constants, monolayers were incubated with [^{3}H]ouabain or [^{3}H]ox-ouabain, (both at 1 μM, 1 μCi/mL) for 30 min. Then the cells were washed four times with 5 mL portions of solution I and incubated in solution I containing either 5×10^{-5} M unlabelled ouabain or ox-ouabain. After various time intervals the medium was

removed, the cells were washed and the radioactivity was determined as above.

To study the concentration dependence of [^{3}H]ouabain or [^{3}H]ox-ouabain binding, monolayers were incubated with various concentrations of the tritiated compound, between 5×10^{-8} M and 5×10^{-6} M, for 30 min at 37°. In all binding experiments, nonspecific binding of [^{3}H]ouabain or [^{3}H]ox-ouabain was determined in the presence of 1 mM of the unlabelled compound, and the results were subtracted from the total binding observed in order to obtain the specific binding. A small series of binding experiments were carried out in the presence of KCl (5 mM) in solution I.

Measurements of cell contractility. The amplitude of systolic cell motion (ASM) and the beating frequencies of individual cells were measured using the phase contrast microscope video motion detector system as described by Biedert *et al.* [21]. The signals were recorded on a strip chart recorder. The changes in the contractility induced by ouabain were calculated and compared with the contractility of the same cells before the addition of ouabain.

Measurements of $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were determined in quin 2-loaded cells as described previously [22]. Rectangular glass coverslips with the attached myocytes were incubated in Ham F10 medium containing HEPES buffer (20 mM, pH 7.4) and quin 2/AM (50 μM), for 1 hr in humidified 5% CO_2–95% air atmosphere at 37°. Additional loading medium (without quin 2/AM) was added for 15 min to complete hydrolysis of quin 2/AM. The cells were then washed with BSS containing (mM): NaCl, 140; KCL, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; Na_2HPO_4, 1; and HEPES, pH 7.4, 10, and incubated in BSS for 30 min. For fluorescent measurement the coverslips with loaded myocytes were inserted into thermostated (37°) cuvette containing 3 mL of BSS. Fluorescence (excitation at 339 nm and emission at 500 nm) was recorded before, and 15 min after the addition of ouabain or ox-ouabain. Values of $[Ca^{2+}]_i$ were calculated as described previously [22].

Measurements of rates of $^{86}Rb^+$ *influx*. Rates of $^{86}Rb^+$ influx were measured according to Panet *et al.*, [23]. Cells plated in culture petri dishes were rinsed with 1 mL of incubation mixture containing (mM): NaCl, 150; RbCl, 5; $MgCl_2$, 5; glucose, 10; $CaCl_2$, 0.5; and HEPES–Tris buffer, 40, pH 7.0. Transport was initiated by the addition of 1 mL incubation mixture at 37° which contained $^{86}Rb^+$ (2 μCi) and ouabain or ox-ouabain at the indicated concentrations. Plates were incubated at 37° for 5 min, and the ^{86}Rb influx was terminated by aspirating the incubation mixture and rinsing the cells twice (rapidly) with 5-mL portions of a solution of $MgCl_2$ (125 mM) and twice with a solution of NaCl (165 mM) at 4°.

The cells were lysed by incubation overnight with 0.6 mL of a solution containing NaOH (0.1 M) and sodium dodecyl sulfate (0.1%, w/v). Aliquots were taken from the solution to determine the radioactivity, in toluene–triton scintillation fluid, and protein content by the method of Lowry *et al.* [20].

In previous experiments it was determined that the $^{86}Rb^+$ uptake into cardiac myocyte culture was linear for 15 min [13, 17]. Therefore in our experiments the initial rate of $^{86}Rb^+$ uptake was calculated from the uptake for 5 min.

"Bumetanide-sensitive" $^{86}Rb^+$ *influx*. $^{86}Rb^+$ influx was measured as above but in the presence and absence of bumetanide (10 μM). The difference between the values obtained represented the "bumetanide-sensitive" $^{86}Rb^+$ influx. The experiments were performed in the absence and presence of ox-ouabain or ouabain at a range of concentrations. The stimulations of the bumetanide sensitive $^{86}Rb^+$ influx by ouabain or ox-ouabain were calculated in comparison with control values obtained in the absence of these drugs in the same experiment. All determinations were done in triplicate.

Measurements of the activity of microsomal ATPase. Microsomal ATPase was prepared from tissue from the cortex and medulla of rabbit or cat kidney according to the procedure described by Jørgensen and Skou [24] as modified by Wald *et al.* [25]. The ATPase activity was determined by the amount of inorganic phosphate (P_i) released during incubation at 37° in a shaking, thermostated bath. The incubation medium consisted of NaCl (100 mM), Tris buffer (50 mM, pH 7.4), $MgCl_2$ (6 mM), KCl (10 mM), EDTA (1 mM), NaN_3 (2 mM). Different concentrations of ouabain or ox-ouabain were added to the reaction mixture. Aliquots of microsomes (10 μg protein) were added to tubes containing 0.5 mL of the reaction mixture, and the suspensions were pre-incubated for 15 min at 37°. The reaction was initiated by the addition of ATP (6 mM) and stopped by the addition of 10% trichloroacetic acid. P_i was determined according to the method of Yoda and Hokin [26].

Analysis of binding data. Association and dissociation rate constants and dissociation constants were obtained from binding data using the non-linear regression computer program P3R of the BMDP statistical software [27]. This program utilizes the Guass–Newton Algorithm to estimate values of fitted parameters that minimize the weighed error sum of squares. The data were weighed according to the inverse variance. A CDC CYBER 855 computer, under the NOS operating system, was used. The values of the standard deviations of all the parameters obtained did not exceed ±7%.

RESULTS

Binding of [^{3}H]*ouabain and* [^{3}H]*ox-ouabain to cultured myocytes*

The kinetics of binding [^{3}H]ouabain and [^{3}H]ox-ouabain, at four different concentrations, to cultured rat myocytes was examined in K^+-free medium during various time intervals between 1 and 60 min. At the higher concentration (5×10^{-6} M) near maximal binding was observed after 5 min for both compounds. At lower concentrations (e.g. 10^{-6}–10^{-7} M), equilibrium binding occurred after longer periods, reaching 15 min with the lowest concentration. Values of $K_{observed}$ were calculated from these binding data according to Kim *et al.* [19]. Non-linear plots indicated the presence of two classes of binding sites for each compound—high and low affinity sites

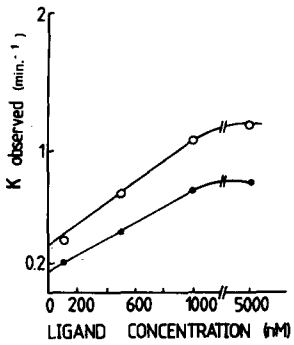

Fig. 2. Plots of $K_{observed}$ vs the concentrations of ouabain or ox-ouabain. Measurements of the time course of binding of ouabain or ox-ouabain at a range of concentrations were used to calculate $K_{observed}$. Binding data (B_t) were plotted as $\ln[B_{max}/(B_{max} - B_t)]$ against time of incubation for each ligand concentration. B_{max} (pmol/mg protein) is the maximal binding after 60 min. The slopes of these lines, which represent $K_{observed}$ were calculated and plotted against the concentrations of ouabain (●) or ox-ouabain (○). Association rate constants (k_a) were calculated from the data. The values obtained by using the computer program P3R nonlinear regression (BMDP statistical software), are presented in Table 1.

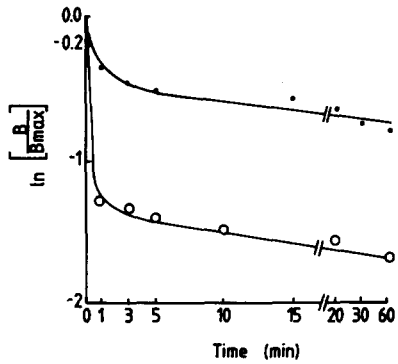

Fig. 3. The time course of dissociation of bound ligand from cultured cardiac myocytes. After binding of [^{3}H]ouabain (●) or [^{3}H]ox-ouabain (○) (1 μM; 1 μCi/mL) for 60 min the media were removed and media containing non-labelled ouabain or ox-ouabain respectively (5×10^{-5} M) were added. The amounts of bound radioactive ligand B, (pmol/mg protein) were determined at different time intervals. The values were used to calculate $\ln(B_{max})$, where B_{max} (pmol/mg protein) is the amount of ligand bound at the beginning of the dissociation ($t = 0$). The non-linear plots indicate the presence of two binding sites for each ligand. The values of the dissociation rate constants k_d, were calculated using a non-linear regression computer program and are given in Table 1. The data show representative experiments. Similar data were obtained in five different experiments, the mean SD was ±5%.

(Fig. 2). Association rate constants (k_a) were obtained from these curves using program P3R for non-linear regression. Higher k_a values were found for ox-ouabain than for ouabain, mainly for the low affinity binding sites (Table 1).

Dissociation rate constants (k_d) were obtained from displacement experiments after binding of either [^{3}H]ouabain or [^{3}H]ox-ouabain (at 10^{-6} M) reach equilibrium. Figure 3 shows plots of the first order integrated rate equation versus time for each compound. Non-linear plots may again result from the presence of the two classes of binding sites. The dissociation rate constants for each class of binding sites for both compounds were calculated using program P3R for non-linear regression. The values of K_D were found to be higher for ox-ouabain than for ouabain, mainly in the low affinity sites (Table 1). Dissociation constants (mean K_D values) calculated from the ratios of the association and dissociation rate constants for each class of binding sites showed similar values for both compounds (Table 1).

The concentration-dependence of equilibrium binding of [^{3}H]ouabain and [^{3}H]ox-ouabain was measured over a concentration range of 10^{-8} to 5×10^{-6} M. Non-linear Scatchard plots of binding

Table 1. Parameters of binding of ouabain and ox-ouabain to cultured cardiac myocytes

Parameters	[^{3}H]Ouabain binding sites		[^{3}H]Ox-ouabain binding sites	
	High affinity	Low affinity	High affinity	Low affinity
Association rate constant k_a (nM^{-1} min^{-1}), 37°	5.3×10^{-4}	1.0×10^{-4}	7.8×10^{-4}	2.4×10^{-4}
Dissociation rate constant k_d (min^{-1}), 37°	0.018	0.8	0.023	2.0
Dissociation constant* $K_D = k_d/k_a$ (M), 37°	3.4×10^{-8}	8×10^{-6}	3×10^{-8}	8.3×10^{-6}
Dissociation constant† K_D (M), 37°	4.2×10^{-8}	3.3×10^{-6}	4.4×10^{-8}	2.9×10^{-6}
B_{max}† (pmol/mg cell protein)	0.2	5.6	0.2	5.6
Sites/cell†	0.48×10^5	1.34×10^6	0.48×10^5	1.34×10^6

* The values were calculated from the association and dissociation rate constants (see Figs 1 and 2).
† The values were calculated from Scatchard plots (see Fig. 3).

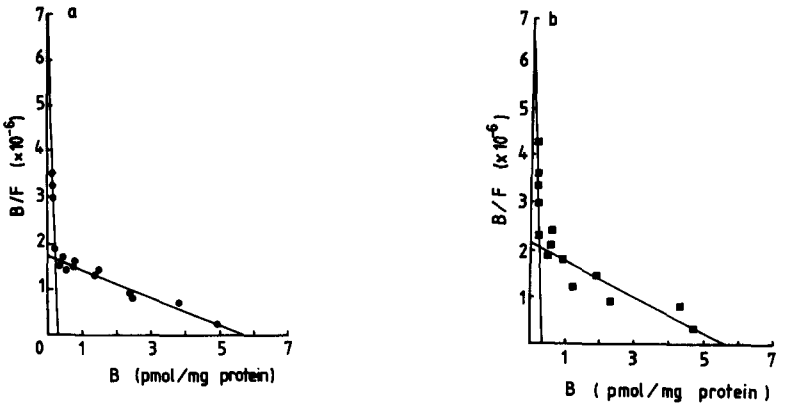

Fig. 4. Scatchard plots of equilibrium binding of ouabain and ox-ouabain. Scatchard plots of the specific binding of ouabain (a) and ox-ouabain (b) were drawn from the 30 min binding data of each of the labelled compounds at a range of concentrations. The specific binding was calculated by subtracting from these binding data the binding in the presence of unlabelled ligand at concentrations of 10^{-3} M. B = bound ligand (pmol/mg protein); F = free ligand (μM). The K_D values of the binding sites were determined using computer program of non-linear regression. The K_D values are presented in Table 1.

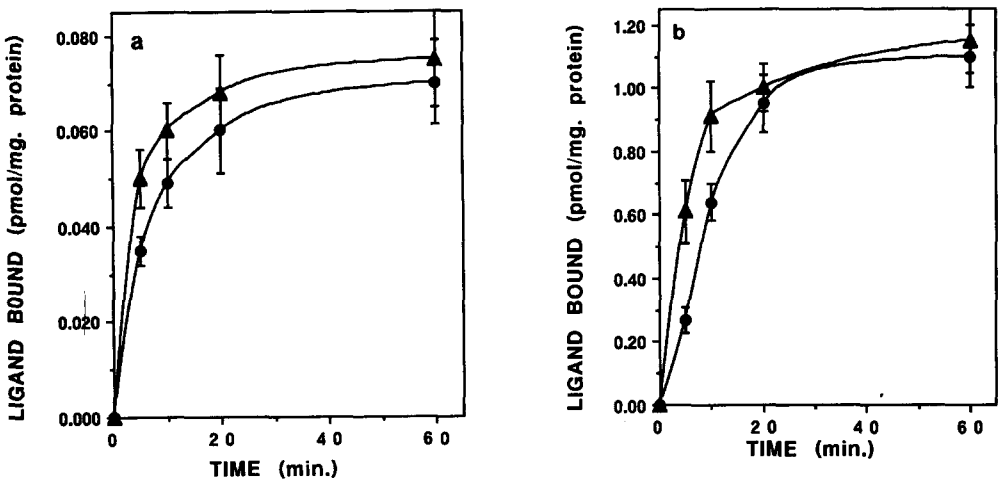

Fig. 5. The time course of binding of [^{3}H]ox-ouabain and [^{3}H]ouabain to cultured myocytes in K^+-containing medium. Binding of the tritiated ouabain (●) and ox-ouabain (▲) was measured as described in Materials and Methods, but KCl (5 mM) was added to solution I. The concentrations of the ligands were (a) 5×10^{-8} M and (b) 10^{-6} M. Values represent means ± SE (N = 3).

augment the motion of the presence of two classes of binding sites for each compound (Fig. 4). The K_D values were similar for ouabain and for ox-ouabain. The numbers of binding sites, calculated from the B_{max} values, were found to be similar for both compounds (Table 1).

The binding experiments described above were conducted in the absence of K^+ from the medium, in order to obtain optimal signal-to-background ratio. However, since changing K^+ in the medium affects ouabain binding, and the K^+-induced decrement in the binding of a specific glycoside may vary among different glycosides, a small series of binding experiments was conducted in a medium which contained 5 mM KCl similar to the media used for physiological measurements. The results in Fig. 5 show that in the presence of 5 mM K^+, ox-ouabain displayed higher rates of association as compared to ouabain. Equilibrium binding of [^{3}H]ouabain and [^{3}H]ox-ouabain have been measured at five different concentrations in the presence of 5 mM KCl. The results in Table 2 indicate similar equilibrium binding for ouabain and ox-ouabain. These binding experiments indicated that the specific features of ox-ouabain binding as compared to ouabain, determined in K^+-free medium, (higher association-rate and similar equilibrium binding) are observed also in the presence of K^+. Higher dissociation rate of ox-ouabain in K^+ containing medium may be deduced from the above results.

To measure the affinity of ox-ouabain to ouabain binding sites, competition experiments were done.

Table 2. Equilibrium binding of [^{3}H]ouabain and [^{3}H]ox-ouabain in the presence of KCl

Ligand concentration (M)	Ouabain bound (pmol/mg protein)	Ox-ouabain bound (pmol/mg protein)
5×10^{-8}	0.070	0.075
10^{-7}	0.120	0.133
5×10^{-7}	0.520	0.540
10^{-6}	1.110	1.150
5×10^{-6}	3.500	3.450

The cultures were incubated in solution I which contained KCl (5 mM) and the tritiated ligands. Equilibrium binding was measured as described in Materials and Methods. The results represent the means of three measurements. The SE values did not exceed ±12%.

Table 3. Displacement of bound [^{3}H]ouabain or [^{3}H]ox-ouabain by unlabelled ouabain and ox-ouabain

Concentration of unlabelled ligand (M)	Bound [^{3}H]ouabain (% of control) in presence of		Bound [^{3}H]ox-ouabain (% of control) in presence of	
	Unlabelled ouabain	Unlabelled ox-ouabain	Unlabelled ouabain	Unlabelled ox-ouabain
0	100	100	100	100
5×10^{-8}	89	92	93	91
10^{-7}	66	69	89	83
5×10^{-7}	41	30	41	51
10^{-6}	25	23	15	13
5×10^{-6}	13	16	13	11
10^{-3}	0	0	0	0

Binding of [^{3}H]ouabain (50 nM, 1 μCi/mL) or [^{3}H]ox-ouabain (50 nM, 0.7 μCi/mL) was measured in presence of unlabelled ouabain or ox-ouabain at the indicated concentration. The binding in presence of 10^{-3} M unlabelled ouabain or ox-ouabain was substracted from the corresponding binding data to obtain specific binding. The specific binding was calculated as percentage of the specific binding of [^{3}H]ouabain or [^{3}H]ox-ouabain without the presence of unlabelled ligand.

In one set of experiments, [^{3}H]ouabain was added to cultured myocytes in the absence and in the presence of various concentrations of unlabelled ox-ouabain (10^{-8}–10^{-3} M). In similar experiments, [^{3}H]ox-ouabain was added to myocytes in the absence and presence of unlabelled ouabain. In addition, [^{3}H]ouabain and [^{3}H]ox-ouabain were added each with its own unlabelled compound (concentrations as above). The results indicate that ouabain and ox-ouabain bind to the same sites, since ouabain displaced ox-ouabain and vice versa, similar to the displacement of each of the labelled compounds by its own unlabelled compound (Table 3).

The effects of ox-ouabain on cell contractility

Amplitude of systolic cell motion (ASM) and beating frequencies of individual cells were examined using a phase contrast microscope video motion detector system [21]. The time course of the effects of ox-ouabain on the ASM (Fig. 6a) was similar to the corresponding time course observed previously in response to ouabain (Fig. 2 in Ref. 22) but the range of concentrations was different. Application of ox-ouabain at concentrations between 5×10^{-8} M and 5×10^{-6} M caused an increase in the ASM and a small decrease in beating frequency without an elevation in the position of maximal relaxation (ΔMR) ("therapeutic range"). Concentrations above 5×10^{-6} M caused a decrease in the ASM, an increase in the spontaneous beating rate and an elevation in the position of maximal relaxation ("toxic range") (Fig. 6). When 5×10^{-8} M ox-ouabain was applied, the ASM increased by 20%, whereas the same concentration of ouabain had no effect. Higher concentrations of ox-ouabain such as 10^{-6} or 5×10^{-6} M still caused positive inotropic effects (50 and 14% increase in the ASM, respectively), whereas similar concentrations of ouabain were in the "toxic range" [22] (Table 4).

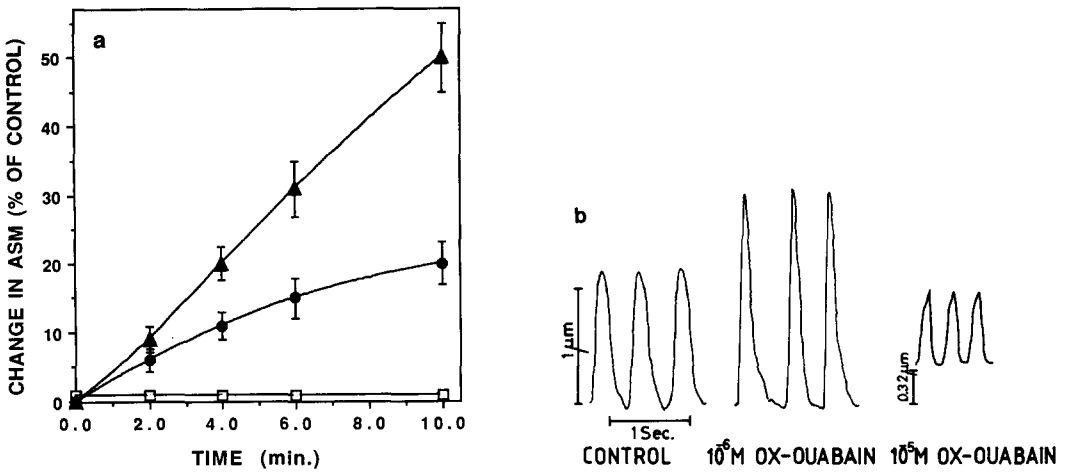

Fig. 6. The effect of ox-ouabain on the cell motion in cultured neonatal rat cardiac myocytes. (a) ASM as relative to the control, was measured as a function of time after exposure to ox-ouabain at the following concentrations: 5×10^{-10} M (□); 5×10^{-8} M (●); or 10^{-6} M (▲). The values indicate means ± SD (N = 5). (b) The traces show steady state motion of a single cell in a monolayer culture perfused with BSS. Measurements were done before (control) and 10 min after the exposure to ox-ouabain (10^{-6} M or 10^{-5} M). Note that the lower dose of ox-ouabain induced an increase in the amplitude of systolic motion without affecting the position of the base line. The higher dose induced a toxic effect manifested by acceleration of spontaneous beating rate with an impaired relaxation and an upward shift of the base line.

Table 4. The effects of ouabain and ox-ouabain on the contractility of cardiac myocytes

Ligand concentration (M)	Amplitude of systolic motion (ASM) (% of control)		Beating frequencies (% of control)		Change in the position of base line (μm)	
	Ouabain	Ox-ouabain	Ouabain	Ox-ouabain	Ouabain	Ox-ouabain
0	100	100	100	100	0	0
5×10^{-8}	100	120 ± 4*	100	96 ± 3	0	0
10^{-7}	127 ± 5*	130 ± 2*	88 ± 4	88 ± 7	0	0
5×10^{-7}	142 ± 2*	138 ± 4*	84 ± 9	92 ± 5	0	0
10^{-6}	46 ± 4*	150 ± 2*	147 ± 5*	83 ± 4	30	0
5×10^{-6}	42 ± 4*	114 ± 6	157 ± 8*	91 ± 12	38	0
10^{-5}	ND	52 ± 2*	ND	142 ± 8*	ND	32

The values represent mean ± SD (N = 5).

The mean ASM and beating frequencies of the control were 1.04 ± 0.02 μm and 137 ± 7.6 beats/min, respectively (means ± SD, N = 10).

* Significant difference from control ($P < 0.05$) by paired *t*-test.

These results indicate that ox-ouabain seems to have a wider "therapeutic range" of concentrations when compared to ouabain as determined in cultured cardiac myocytes.

The time course of reactivation after a "toxic dose" of ouabain or ox-ouabain

The time course of reactivation of cell contractility after inhibition by a toxic concentration of ouabain (5×10^{-6} M) or ox-ouabain (10^{-5} M) was determined. Ouabain or ox-ouabain were applied to cells for 15 min, and by that time the contractility had decreased. Changing to drug-free medium caused gradual reactivation of contractility. The time course of this reactivation was different if the cells were exposed to ouabain or ox-ouabain. After 1 min of washing out, the cells treated with ox-ouabain gained 80% of their control-level contractility, whereas cells treated with ouabain gained only 20%. After 10 min of washing out, the cells treated with ox-ouabain were reactivated to 93% of the control level as compared with 60% in cells treated with ouabain (Fig. 7).

The time courses of contractility reactivation by washing out toxic doses of ouabain or ox-ouabain were compared to time courses of the release of these drugs bound to myocytes. Cells were incubated for 30 min with [^{3}H]ouabain or [^{3}H]ox-ouabain

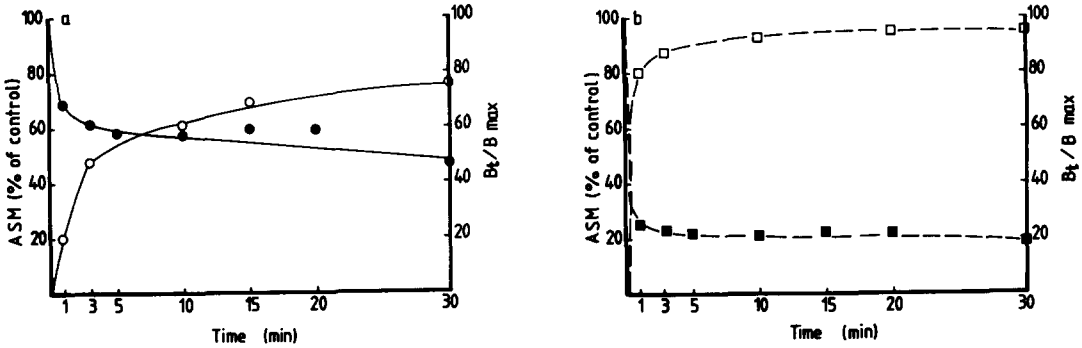

Fig. 7. The reversibility of the toxic effect of ouabain and ox-ouabain, and the time courses of dissociation of bound ligand. The reactivation of cells after exposure to a toxic dose of (a) ouabain (10^{-5} M) or (b) ox-ouabain (5×10^{-5} M), was measured during perfusion with ligand-free medium. The ASM was measured before the addition of the toxic dose (control), and during the course of the washout. The values are expressed as per cent of the control level of the same cell before the addition of the toxic dose of ouabain (○) or ox-ouabain (□). Cells were exposed to (a) [^{3}H]ouabain or (b) [^{3}H]ox-ouabain (10^{-6} M, 1 μCi/mL). At $t = 0$ the medium was removed and the cells were perfused by non-labelled ouabain (●) or ox-ouabain (■) respectively (5×10^{-5} M). The cell-bound radioactivity (B_i) was measured at different time intervals during the washout and expressed as a fraction of the maximal radioactivity at $t = 0$.

Table 5. Changes in steady-state levels of $[Ca^{2+}]_i$ in cardiac myocytes exposed to ouabain and ox-ouabain

	Concentration	$[Ca^{2+}]_i$ (nM)
Control	—	141 ± 8
Ouabain	5×10^{-7} M	237 ± 11
Ouabain	5×10^{-6} M	264 ± 14
Ox-ouabain	5×10^{-7} M	205 ± 7
Ox-ouabain	5×10^{-6} M	236 ± 7

$[Ca^{2+}]_i$ was calculated from fluorescence measurements in quin-2 loaded cells before, and 15 min after the addition of ouabain or ox-ouabain.

(10^{-6} M). By this time binding equilibrium was achieved. The media were then replaced with media containing non-radioactive ouabain or ox-ouabain respectively (5×10^{-5} M), and the time course of the decrease in the cell associated radioactivity was measured. Figure 7 shows that the washout of [^{3}H]ox-ouabain was much faster than that of ouabain; in 1 min 75% of the cell associated radioactivity was released as compared with 33% of the bound [^{3}H]ouabain. These results may indicate that faster reactivation of cells treated with ox-ouabain is due to a faster exchange of the bound drug, probably due to its higher dissociation rate constant of the low affinity site (Table 1).

Effects of ox-ouabain on $[Ca^{2+}]_i$

Ox-ouabain causes a steady state elevation in the level of $[Ca^{2+}]_i$, as measured by the fluorescence intensity in quin 2-loaded cells (Table 5). The increase in $[Ca^{2+}]_i$ by concentrations of 5×10^{-7} and 5×10^{-6} M ox-ouabain was below the increase induced by similar concentrations of ouabain.

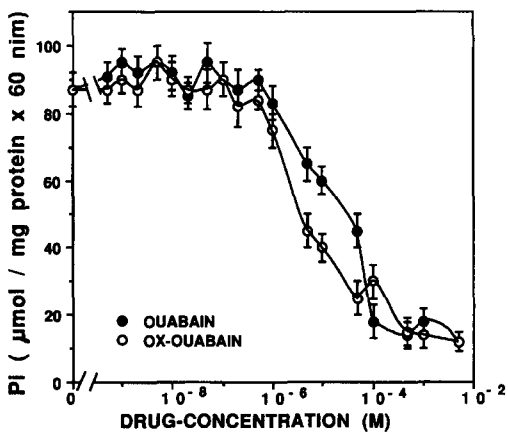

Fig. 8. Effects of ouabain and ox-ouabain on the activity of Na^+, K^+-ATPase. Effects of ouabain and ox-ouabain on the activities of microsomal Na^+, K^+-ATPase, prepared from cat-kidney, were examined at a range of concentrations as described in Materials and Methods. Activities were determined by the amounts of inorganic phosphate (P_i) released during 60 min incubation. Values represent means ± SD (N = 3).

The effect of ox-ouabain on the activity of Na^+,K^+-*ATPase*

The inhibiting effects of ox-ouabain and ouabain on the activity of Na^+,K^+-ATPase were compared (Fig. 8). At the lower concentration range (5×10^{-10}–10^{-6} M) neither compound exerted any affect on Na^+,K^+-ATPase activity. At the higher concentration range (10^{-6}–10^{-3} M), dose dependent inhibitory effects were exerted by both compounds. Ox-ouabain was slightly more potent than ouabain at concentrations between 5×10^{-6} and 5×10^{-5} M.

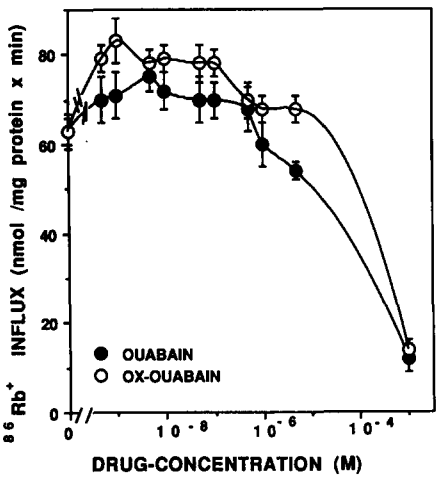

Fig. 9. Effects of ouabain and ox-ouabain on the rates of $^{86}Rb^+$ influx. The effect of different concentrations of ouabain and ox-ouabain on the rates of $^{86}Rb^+$ influx into cultured rat cardiac myocytes were measured during 5 min at 37° as described in Materials and Methods.

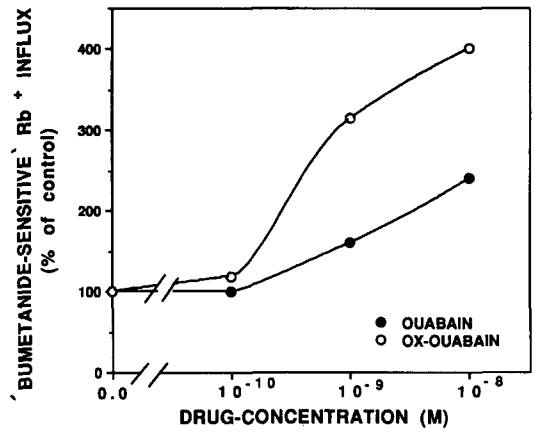

Fig. 10. Effects of ouabain and ox-ouabain on the "bumetanide-sensitive" $^{86}Rb^+$ influx. Rates of $^{86}Rb^+$ influx were measured during 5 min at 37° in the absence and presence of bumetanide. The differences between the values represented "bumetanide-sensitive" $^{86}Rb^+$ influx. Results are expressed as "bumetanide-sensitive" $^{86}Rb^+$ influx measured in the presence of different concentrations of the drugs (ouabain or ox-ouabain) as a percentage of the "bumetanide-sensitive" $^{86}Rb^+$ influx measured in the absence of the drugs in the same experiment. The mean control value of bumetanide-sensitive Rb^+ influx is 4.9 ± 0.2 nmol/min · mg protein.

Effects of ox-ouabain on rates of $^{86}Rb^+$ influx

The effects of ox-ouabain and ouabain on the rates of $^{86}Rb^+$ (analog of K^+) influx were found to be bi-phasic (Fig. 9). At low concentrations of ouabain and ox-ouabain, stimulation of $^{86}Rb^+$ influx rates were observed, whereas high concentrations of both substances inhibits $^{86}Rb^+$ influx. Significant stimulation ($P > 0.05$) were obtained in the presence of 5×10^{-9} M and 10^{-8} M ouabain. Concentrations of ouabain above 10^{-6} M inhibited $^{86}Rb^+$ influx. The stimulation of $^{86}Rb^+$ influx in the presence of low concentrations of ox-ouabain was more pronounced. Significant stimulations of $^{86}Rb^+$ influx were observed in the presence of ox-ouabain between 5×10^{-10} M and 10^{-7} M ($P > 0.05$ by *t*-test). Inhibition of $^{86}Rb^+$ influx was observed only at above 5×10^{-6} M ox-ouabain. Inhibition of $^{86}Rb^+$ influx by 50% was obtained with 2×10^{-4} M and 6×10^{-4} M ouabain and ox-ouabain, respectively. "Bumetanide sensitive" $^{86}Rb^+$ influx has been determined at the lower concentration range (Fig. 10). Ox-ouabain at 10^{-9}, induced a 3.15-fold stimulation of "bumetanide sensitive" $^{86}Rb^+$ influx as compared with 1.60-fold stimulation by ouabain. Concentrations of 10^{-8} M ouabain and ox-ouabain induced stimulation by 2.44- and 4.02-fold of the "bumetanide sensitive" Rb^+ influx, respectively. It is concluded that ox-ouabain is more potent in stimulating the Rb^+ influx via the bumetanide sensitive cotransporter. Bumetanide resistant $^{86}Rb^+$ influx was not altered by concentrations of ouabain and ox-ouabain between 10^{-10} and 10^{-8} M (not shown).

DISCUSSION

Investigations on the effects of ouabain on cultured chick heart cells convincingly indicated a direct relation between ouabain binding, inhibition of the Na^+, K^+-pump, the rise in $[Na^+]_i$ and the increase in contractile force [19, 28]. One single class of receptors for ouabain was characterized in chick heart muscle cells and in cultured chick heart myocytes [6, 7], whereas in cardiac cells from mammals two classes of receptors for cardiac glycosides were detected: in the heart muscle cells from neonatal rats high affinity sites ($K_D = 3.2 \times 10^{-8}$ M) and low affinity sites ($K_D = 7.1 \times 10^{-6}$ M) were both characterized [8]. Similarly, two binding sites for cardiac glycosides were detected in cultured neonatal rat cardiac myocytes [29], in bovine and human cardiac Na^+,K^+-ATPase [10] and in dog cardiac myocytes [30].

It appears that the mechanisms of action of cardiac glycosides in species having two classes of binding sites is more complex. The main difficulty in the interpretation of the results is caused by stimulation of K^+ influx, rather than inhibition, by concentrations of ouabain that cause a positive inotropic effect [15].

Recent study in our laboratories has led to a suggestion which may resolve the controversy [13]. At low concentrations, the component of $^{86}Rb^+$ influx which is stimulated by ouabain is inhibited by bumetanide. Since bumetanide is an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter it appears that low concentrations of ouabain stimulate the activity of this cotransporter. Stimulation would appear to lead to enhanced influx of K^+ and Na^+, increased $[Na^+]_i$ and, via the Na^+/Ca^{2+} exchanger, to an increase in $[Ca^{2+}]_i$ and contractile force. Further investigations have established that bumetanide abolished the positive inotropic affect induced by a low concentration of ouabain. The ouabain induced elevation in $[Na^+]_i$ was also inhibited by bumetanide [16].

In the present work we proceed to examine the mechanism of action of cardiac glycosides in cultured neonatal rat cardiac myocytes by comparing the

effect of ouabain to the effect of a new derivative of ouabain obtained by mild oxidation. A procedure for chemical modification of ouabain by oxidation with $NaIO_4$ has been published previously [31], but the biological activity of the modified derivative has not been determined. In the present work, ouabain was oxidized under milder conditions resulting in a selective cleavage of the bond between C2′ and C3′ of the sugar moiety but caused no effect on the steroid moiety, as determined by ^1H NMR spectroscopy [17].

Displacement measurement indicated that oxidized ouabain binds to ouabain receptors. The nature of the sugar moiety is known to affect mainly the dissociation rate constant [32]. In the present work, cleavage between C2′ and C3′ was found to cause higher rate constants of association and dissociation mainly of the low affinity sites. On the other hand, as both association and dissociation rate constants of ox-ouabain increased to a similar extent, the dissociation constants (K_D), calculated from their ratio, were not significantly different from those of ouabain. These results were confirmed by direct measurements of K_D by Scatchard analysis. Two classes of binding sites, of a high and a low affinity, were detected for both ouabain and ox-ouabain. A small series of experiments conducted in a medium containing 5 mM KCl (similar to the media in physiological experiments) indicated that the above pattern of differences in binding between the two ligands is not altered in the presence of K^+.

Our results therefore show that ox-ouabain differs from ouabain in turnover rate of binding, mainly of the low affinity site, but is similar to ouabain in its affinity to its receptor. These results led us to examine whether the biological effect of the glycoside is determined only by the affinity of the drug to its receptor or in conjunction with other properties such as the turnover rate of binding. It has been shown that the inhibition of Na^+,K^+-ATPase is determined by the affinity of the drug to the receptor, but the turn-over rates affect the biological response to the drug in guinea pig heart preparations [33]. To examine this notion we have compared the effects of ouabain and ox-ouabain on the activity of Na^+,K^+-ATPase and on inducing a biological response. A similar profile of inhibition of Na^+,K^+-ATPase was detected. At the lower concentration range (10^{-10}–10^{-6} M) neither compound inhibited the activity of the enzyme.

The biological effects of ouabain and ox-ouabain were estimated from their induced change in the ASM. Whereas the positive inotropic effect was manifested by an increase in ASM, a "toxic effect" was characterized by a decrease in ASM, increased beating frequency and an elevation of the position of the maximal relaxation [22]. It was found that the inotropic effect induced by ox-ouabain was characterized by a broader dose–response curve that initiated at a lower concentration than ouabain. The inotropic effect was induced by concentrations which did not inhibit the activity of Na^+,K^+-ATPase in microsomal preparation. These results are in accordance with the findings of Lullman *et al.* [33] which showed that glycosides which display a narrow mechanical dose–response curve had a slow turnover rate of binding, whereas semi-synthetic glycosides which are characerized by a broad dose–response curve and by a greater magnitude of inotropic response possess a fast turnover rate of binding.

Influx of $^{86}Rb^+$ into cardiac myocytes is a measure of the sum of activities of two classes of transporters: Na^+,K^+-ATPase and the $Na^+/K^+/2Cl^-$ cotransporter, in addition to a component of non-specific diffusion. The activity of the cotransporter is inhibited by loop diuretics such as bumetanide and furosamide [34–36] and therefore is determined by measuring the fraction of $^{86}Rb^+$ influx which is inhibited by bumetanide, i.e. "bumetanide sensitive" $^{86}Rb^+$ influx.

In the absence of ouabain, $^{86}Rb^+$ influx via the cotransporter is about 10% of the total influx. A low concentration of ouabain (10^{-8} M) stimulates the influx via the cotransporter by 2.4-fold [13]. It was found in the present work that the stimulation of the bumetanide sensitive $^{86}Rb^+$ influx by ox-ouabain was much higher than by ouabain, and was induced at a lower concentration range. These findings may explain the positive inotropic effect of ox-ouabain at lower concentrations than ouabain. Although stimulation of "bumetanide sensitive" $^{86}Rb^+$ influx by ox-ouabain could be detected at somewhat lower concentrations (10^{-9} M) than the inotropic effect (5×10^{-8}) this discrepancy may be attributed to the lower sensitivity of the method of measuring ASM as compared to the $^{86}Rb^+$ influx. The nature of the interaction which leads to the higher stimulation of the cotransporter by ox-ouabain remained unresolved.

At the higher concentration range, above 10^{-6} M, ouabain induced a toxic effect, whereas ox-ouabain showed inotropic effect with no signs of toxicity up to a concentration of 10^{-5} M. These differences may perhaps be attributed to the somewhat lower steady state level of $[Ca^{2+}]_i$ following the addition of ox-ouabain, as compared with ouabain. However, a recent study has indicated that the glycoside-mediated cardiac toxicity is more complex than simple elevation of $[Ca^{2+}]_i$, as measured with fura 2 [37]. It would be reasonable to suggest that the reduced toxicity of ox-ouabain may be linked with the faster reversibility of ox-ouabain binding to the low affinity site.

In conclusion, this work indicates, in accordance with our previous studies [13, 16, 17, 22] that the mechanism of action of digitalis glycosides in cardiac myocytes from neonatal-rat cannot be satisfactorily explained by the model postulating direct relations between pump inhibition and increased contractile force. We suggest that the glycosides exert two independent actions on mammalian cardiac cells. (i) At the higher concentration range, inhibition of the Na^+,K^+-ATPase is the main pathway leading to the inotropic or toxic effect. The toxic effect may be affected by the extent of reversibility of drug-binding to the low affinity site. (ii) At the lower concentration range, the positive inotropic effect seems to be mediated via stimulation of the $Na^+/K^+/Cl^-$ cotransporter.

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