REVIEWS

Cardiac Lysosomes and the Mechanism of Action of Ouabain

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Cardiac glycosides are the main cardiotonic preparation used in the treatment of acute and chronic cardiac failure. So far, the mechanism underlying the effect of cardiac glycosides is not fully understood. It has been hypothesized that their effect is associated with inhibition of Na^+,K^+-ATPase in the cardiomyocyte plasma membrane [20]. Two binding sites for ^3H-ouabain colocated with transport ATPase have been identified [9]. Other researchers believe that of Na^+,K^+-ATPase is inhibited only by toxic doses of cardiac glycosides, and new glycoprotein with high affinity for ouabain does not depend on transport ATPase [11]. Cardiotonic effect of cardiac glycosides may be related to their interaction with intracellular structures [14]. Cardiac glycosides were detected in the area of contractile proteins, sarcoplasmic reticulum, and nucleus [10]. It was shown that the binding sites for ^3H-ouabain on the plasma membrane of HeLa cells are cleared by dissociation of the ligand in the culture medium and by its internalization. Ouabain is located in lysosomes [3]. Similar results were obtained in experiments on isolated heart [18].

Cardiac lysosomes are involved in a number of important physiological processes, including autophagy, cardiomyocyte regeneration, endocytosis, and intracellular transport of drugs and hormones [2,8, 12,13,22]. They participate in the development of pathological processes. Increased permeability of lysosomal membranes and release of acid hydrolases in the cytosol may cause myocardial damage [23]. The effect of ouabain on activity of lysosomal enzymes in the heart has not been investigated.

Our objective was to find out how the effect of ouabain on activity of myocardial acid hydrolases depends on the dose and time after administration. We also examined the effect of this cardiac glycoside on cardiomyocyte lysosomes *in vitro*.

MATERIALS AND METHODS

Experiments were performed on outbred male albino rats (body weight 140-160 g) maintained under standard vivarium conditions. Ouabain was administered intraperitoneally in a single doses of 0.1, 1, 6, 12, 24, or 50 mg/kg. The rats were decapitated under light ether anesthesia after 5 and 30 min and 2, 4, and 24 h. The heart was rapidly removed an homogenized in a Teflon:glass homogenizer on the cold (2 min, 1500 rpm) in medium containing 0.25 M sucrose, 0.001 M EDTA in 0.05 M Tris-HCl buffer (pH 7.4) [7]. Lysosomes were isolated from the homogenate by differential centrifugation [21]. In *in vitro* experiments lysosomes were incubated with 10^{-8}, 10^{-6} and 10^{-3} M ouabain for 30 min at 37°C.

Activities of β-glucosidase (EC 3.2.1.21) and β-galactosidase (EC 3.2.1.23) were measured by the method [19]. This method is based on the ability of 4-nitrophenol, which is formed upon hydrolysis of synthetic substrates by lysosomal glycosidases, to produce characteristic yellow coloration in alkaline medium with maximum light absorbance at 420 mM. 4-Nitrophenyl-β-D-glycopyranoside and 4-nitrophenyl-β-D-galactopyranoside were used as substrates. Activity of

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lysosomal acid phosphatase (AP, EC 3.1.3.2) was measured spectrophotometrically using sodium β-glycerophosphate as a substrate [4]. This method is based on the ability of phosphoric acid, which is released upon hydrolysis of sodium β-glycerophosphate, to form a complex with ammonium molybdate. Reduction of this complex by ascorbic acid yields dark blue coloration with the maximum light absorbance at 750 mM. The accessibility of substrate for enzyme was estimated as the ratio between free and total activities of acid hydrolases. Total activity was determined with the use of Triton X-100 in a final concentration of 0.1% (β-galactosidase and AP)-and 0.05% (β-glycosidase).

Activity of the enzymes was expressed in μmol/min×g protein.

Protein content was determined by the method of Lowry [16]. The results were analyzed using Student's *t* test [1].

RESULTS

In doses 0.1, 1, and 6 mg/kg ouabain had practically no effect on myocardial lysosomes.

In a dose of 12 mg/kg it modified activities of lysosomal enzymes. Free β-glycosidase activity decreased 5 min after its administration to 73.4±5.4% of the control (Table 1). Then the enzyme activity was restored and did not differ from the control throughout the entire observation period. A 25% decrease in total β-glycosidase activity was observed 4 h after administration of ouabain.

Activity of β-galactosidase was inhibited to a greater extent than that of β-glycosidase: 5 min after administration of ouabain free and total activities decreased by 34.9 and 39%, respectively. After 30 min free activity returned to the control level and remained unchanged throughout the entire observation period. Total enzyme activity was decreased 30 min and 2 and 24 h after administration of ouabain.

After administration of ouabain in a dose of 12 mg/kg free and total AP activities remained unchanged throughout the entire observation period.

Ouabain produced the strongest inhibitory effect on the activity of myocardial lysosomal enzymes in a dose of 24 mg/kg. Table 2 shows the dynamics of free and total β-glucosidase activities. Five minutes after administration of ouabain, free β-glucosidase and total β-glucosidase activities decreased 3-and 2-fold, respectively. The enzyme was inhibited throughout the entire observation period. Free activity of

TABLE 1. Activity of Lysosomal Enzymes in Rat Heart after Administration of 12 mg/kg Ouabain (% of Control, *M±m*)

Time	Free activity	Total activity	Free/total, %
β-glucosidase			
Control	100.0±3.9	100.0±2.8	100.0±5.4
5 min	73.4±5.4*	96.0±15.7	79.7±9.2
30 min	86.2±11.8	89.4±5.0	94.9±4.6
2 h	89.9±4.5	96.8±12.7	97.2±3.1
4 h	81.4±8.0	75.0±8.3*	108.2±9.3
24 h	95.9±8.4	106.5±10.2	90.7±7.8
β-galactosidase			
Control	100.0±4.3	100.0±3.8	100.0±4.4
5 min	65.1±9.6*	61.0±1.8*	105.2±13.1
30 min	89.4±6.3	76.0±5.0*	120.0±9.6
2 h	80.5±15.7	74.5±5.9*	98.8±11.9
4 h	82.8±10.0	81.1±12.2	108.1±14.4
24 h	82.0±8.9	71.0±6.4*	110.3±3.9
AP			
Control	100.0±3.3	100.0±6.5	100.0±5.1
5 min	89.7±6.5	90.8±10.5	102.5±8.3
30 min	90.0±5.2	82.6±9.1	107.1±4.5
2 h	86.8±7.4	89.5±3.6	102.0±7.1
4 h	95.5±4.2	95.7±4.3	100.0±3.6
24 h	88.5±7.6	90.1±3.8	98.6±5.1

Note. Here and in Tables 2 and 3: *$p<0.05$ compared with the control; values are the means of 6-10 determinations.

TABLE 2. Activity of Myocardial Acid Hydrolases after Administration of 24 mg/kg Ouabain (% of Control, *M±m*)

Time	Free activity	Total activity	Free/total, %
β-glucosidase			
Control	100.0±12.8	100.0±8.9	100.0±7.0
5 min	38.7±3.2*	50.0±2.9*	77.0±3.7*
30 min	32.9±7.5	49.2±1.5*	79.3±3.9*
2 h	40.3±1.9*	46.6±3.2	86.5±4.4
4 h	40.3±1.8*	46.6±3.1*	86.5±4.3
24 h	62.7±2.9*	75.1±2.4*	86.3±1.6
β-galactosidase			
Control	100.0±12.7	100.0±11.6	100.0±11.9
5 min	19.9±3.0*	24.32±0.9*	81.5±3.1
30 min	9.8±8.9*	29.2±3.3*	33.4±7.4*
2 h	39.3±2.1*	36.3±2.1*	106.3±7.4
4 h	55.3±1.4*	59.1±1.0*	102.8±2.1
24 h	92.7±1.0	107.9±0.8	85.3±1.2
AP			
Control	100.0±11.9	100.0±8.3	100.0±4.6
5 min	67.1±2.9*	77.9±2.9*	85.7±1.7
30 min	65.3±2.9*	75.2±0.8*	86.0±2.3*
2 h	70.6±1.2*	65.7±3.5*	112.5±4.2
4 h	53.5±2.4*	56.2±2.6*	95.5±2.8
24 h	70.4±3.4*	56.6±3.5*	123.6±1.3*

β-glucosidase was minimal (32.9±7.5% of the control) 30 min after administration of ouabain, while the minimal total activity was recorded on the 2nd and 4 h of observation period. After 24 h, free and total activities slightly increased without reaching the control level. As a result of strong inhibition of free β-glucosidase activity on the 5th and 30th min of observation period, the percent ratio of free to total activity decreased, indicating that ouabain affects lysosomal membranes at these terms of observation period.

In a dose of 24 mg/kg ouabain had a stronger effect on β-galactosidase than on β-glucosidase activity. Five minutes after administration of ouabain, free activity and total activities decreased 5- and 4-fold, respectively, compared with the control. Free activity further decreased, amounting only for 9.8±8.9% of the control level on the 30th min. At the same term of investigation the percent ration between free and total β-galactosidase activities decreased, indicating that ouabain diminished the accessibility of the enzyme for the substrate. After 2 and 4 h, free and total β-galactosidase activities increased, reaching the original level after 24 h.

Ouabain had a weaker inhibitory effect on AP activity than on activities of β-galactosidase and β-glucosidase. Five minutes after its administration, free AP activity was 67.1±2.9% and total activity was 77.9± 2.9% of the control. The enzyme activity remained unchanged after 30 min and 2 h; however, after 4 h both— and free and total enzyme activities dropped to 53.5±2.4 and 56.2±2.6%, respectively. After 24 h free AP activity slightly increased (70.4±3.4% of the control), while total activity remained at the same level, which was reflected by increased percent ratio between free and total activities (123.6±1.3%).

Thus, in a dose of 24 mg/kg ouabain inhibited all studied acid hydrolases and reduced their accessibility for the substrate. β-Galactosidase proved to be most sensitive to this preparation: 30 min after its administration free enzyme activity dropped 10-fold in comparison with control. Ouabain produced a weaker inhibitory effect on β-glucosidase than on β-galactosidase, the effect being minimal in case of AP. These modifications were prolonged and manifested themselves 24 h after after administration of the preparation.

Two-phase modulation of enzyme activities was observed after administration of 50 mg/kg ouabain. After 5 min, both free and total β-glucosidase activities decreased to 26.8±4.1 and 57.4±7.7% of the control (Table 3). Accordingly, the free/total activity

TABLE 3. Activity of Myocardial Lysosomal Enzymes after Administration of 50 mg/kg Ouabain (% of Control, *M±m*)

Time	Free activity	Total activity	Free/total, %
β-glucosidase			
Control	100.0±2.1	100.0±2.9	100.0±2.4
5 min	26.8±4.1*	57.4±7.7*	50.0±10.3*
30 min	94.7±10.2	92.3±9.6	110.9±22.3
2 h	178.0±16.2*	151.8±17.9*	123.1±12.0
4 h	128.8±20.5	170.8±8.9*	76.3±13.0
24 h	100.0±11.7	101.0±7.6	98.1±7.8
β-galactosidase			
Control	100.0±2.3	100.0±3.0	100.0±2.00
5 min	46.1±10.8*	59.0±2.7*	95.3±17.1
30 min	260.3±18.3*	338.7±41.0*	87.4±15.9
2 h	198.2±9.1*	200.0±15.2*	98.1±11.2
4 h	130.5±7.8*	150.4±15.6*	86.8±9.90
24 h	108.2±9.2	112.0±10.3	96.5±12.1
AP			
Control	100.0±6.4	100.0±5.0	100.0±3.0
5 min	155.3±9.9*	155.9±10.3*	99.8±2.9
30 min	103.6±12.7	110.1±13.5	93.9±2.0
2 h	87.4±3.9	72.9±3.7*	132.7±7.6*
4 h	85.6±2.7	76.8±4.8*	112.1±6.8
24 h	94.9±8.6	102.7±8.1	92.9±4.7

ratio decreased, indicating low substrate accessibility of the enzyme. Both activities were normalized after 30 min and increased, respectively, to 78.0 and 51.8% of the control. Then free activity was normalized, while total activity rose, reached the maximum after 4 h (170.8±8.9% of the control), and returned to the initial level after 24 h.

Ouabain also suppressed β-galactosidase activity: 5 min after its administration free and total enzyme activities dropped more than 2-fold and 3-fold, respectively, compared with the control. However, the enzyme activity sharply increased after 30 min: free activity amounted for 260.3±18.3% and total activity 338.7±41.0% of the control. Then it gradually decreased (after 2 and 4 h) and returned to the initial level after 24 h.

In contrast to β-glucosidase and β-galactosidase activities, AP activity increased more than 50% 5 min after administration of 50 mg/kg ouabain. Throughout the further observation period free AP activity did not differ from the control. Total AP activity gradually decreased, being significantly lower than in the control after 2 and 4 h. It was normalized after 24 h.

Thus, in a dose of 50 mg/kg ouabain stimulated all studied lysosomal enzymes. Glucosidase activity rose after a drop. β-Galactosidase proved to be most sensitive to 50 mg/kg ouabain. The preparation had a weaker stimulatory effect on β-glucosidase. The lowest stimulating effect was observed with AP. The nature and intensify of ouabain effect on lysosomal enzymes of the myocardium depended on dose, time after administration, and individual sensitivity of some acid hydrolases to an cardiac glycoside to an ouabain. In a dose of 12 mg/kg glucosidases were inhibited at the same terms of observation period. At 24 mg/kg, ouabain strongly inhibited all studied enzymes, decreased their accessibility for substrates. At 50 mg/kg it exerted a two-phase effect: first inhibited and then stimulated glycosidase activities and, on the contrary, first stimulate and then inhibited AP. Most pronounced changes in enzyme activities were observed 30 min (β-galactosidase), 2 h (β-glucosidase), and 4 h (AP) after administration of ouabain. These changes were the stron-gest with β-galactosidase and the weakest with AP.

As we are aware, there is no published data on the effect of ouabain on activity of acid hydrolases of the myocardium. The information regarding internalization and accumulation of the glycoside in lysosomes [3,18] points to its direct effect.

Incubation of lysosomal fraction of the myocardium with 10^{-8}, 10^{-6}, and 10^{-3} M ouabain at

37°C for 30 min markedly modified the activities of lysosomal enzymes. At 10^{-8} M ouabain deceased free and total β-glucosidase activities, had no effect at 10^{-6} M, and increased free activity at 10^{-3} M.

At 10^{-8} M ouabain inhibited β-galactosidase and stimulated it at 10^{-3} M.

At 10^{-8} M ouabain increased free AP activity, the effect being maximal at 10^{-3} M. Total AP activity remained unchanged, which led to an increase in the free/total activity ratio, indicating that the accessibility of AP for substrate increased.

Thus, ouabain exerts direct effect on myocardial lysosomes. It lowers free and total activities of glycosidases in a concentration of 10^{-8} M and elevates it in a concentration of 10^{-3} M. Our results demonstrate that ouabain elicits similar effects on the activity of acid hydrolases *in vivo* and *in vitro*, which confirms the data on internalization and accumulation of cardiac glycosides in lysosomes.

There is no published data on the mechanism underlying the inhibitory effect of ouabain on lysosomal enzymes. It was demonstrated that the activity of β-glycosidase, β-galactosidase, and AP depends on the integrity of SH-groups. Compounds blocking SH-groups act as inhibtiors of lysosomal enzymes [4]. Ouabain is a blocker of SH-groups; due to the presence of unsaturated lactonic ring it oxidizes SH-groups of Na,K ATPase [15]. Therefore, it can be suggested that inhibition of lysosomal enzymes by ouabain is associated with intralysosomal blockade of their SH-groups.

Our findings suggest that inhibition of acid hydrolases of the myocardium is a consequence of the following events: ouabain enters the cell by receptor-dependent endocytosis and is accumulated in the cytoplasm in the form of endosomes. Then the endosomes fuse with lysosomes, and ouabain inhibits lysosomal enzymes by blocking their SH-groups.

Different localization of acid hydrolases may account for their different sensitivity to ouabain. Glycosidases are located predominantly in the lysosomes of cardiomyocytes, while AP is concentrated in the lysosomes of interstitial cells [23]. β-Glucosidase predominates in membrane-bound form, while β-galactosidase is a matrix enzyme of lysosomes [7].

Contractile function of the heart strongly depends on integrity of intracellular structures. In some diseases this function is impaired when degradation of intracellular structures prevails over their synthesis [6]. This may be associated with increased number of lysosomes in the heart, increased permeability of lysosomal membranes, and release of acid hydrolases in the cytosol [5]. In fact, lysosomal enzymes degrade myofibrils and mitochondrias [8] and inhibit myofibrillar Ca^{2+} ATPase.

Proceeding from these considerations, inhibition of β-glucosidase, β-galactosidase, and AP by 12 and 24 mg/kg ouabain probably reflects physiological modifications of myocardial lysosomes as a component of the mechanism of positive inotropic effect of this cardiac glycoside. By contrast, sharp increase in the activity of acid hydrolases caused by toxic dose of the preparation (50 mg/kg) is an unfavorable factor promoting the development of focal necrosis and dystrophy, which was observed in intoxication with cardiac glycosides [17].

Thus, ouabain produces a dose-dependent effect on activity and latency of lysosomal enzymes of heart. At 12 and 24 mg/kg it lowers the activity of acid hydrolases and their accessibility for the substrate. At 50 mg/kg it exerts two-phase effect: first suppresses, then raises glycosidase activity of and first raises and then lowers AP activity.

Ouabain has direct effect on myocardial lysosomes. In a concentration of 10^{-8} M it inhibits glycosidase activity, while in a concentration of 10^{-3} M it stimulates all studied acid hydrolases.

According to the sensitivity to ouabain, lysosomal enzymes can be arranged as follows: β-galactosidase>β-glucosidase>acid phosphatase.

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