Effect of Ouabain on Actomyosin—Studies on the Effect of Ouabain on Actomyosin Extracted from Cardiac, Both Normal and Failing, and Skeletal Muscles

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This report deals with the role played by cardiac glycosides on the reaction between adenosin triphosphate (ATP) and Actomyosin (AM) isolated from dog's heart muscles, both normal and failing, and also from rabbit psoas muscles. All of physicochemical properties examined, i. e., superprecipitation, AM-ATPase activity, and isometric tension-development of glycerinated fibers were inhibited by high concentrations of ouabain. Low concentration of ouabain accelerated superprecipitation, and unaffected ATPase activity and tension-development (in Ca-free medium). In Ca containing medium, the inhibitory effect of Ca on tension-development tended to be eliminated by low concentrations, and was augmented by high concentrations of ouabain. Thus Ca has some relationship with the action of ouabain on membrane-free contractile protein. The impaired properties of AM extracted from dog's hearts with right-sided heart failure were not improved by ouabain, and there were no fundamental differences in the actions of this drug between AM prepared from normal cardiac, skeletal, and failing cardiac muscles.

ALTHOUGH numerous studies have uncovered many interesting facts in the action of cardiac glycosides, the mechanism of the ultimate cellular action still remains unknown. It is now generally agreed, however, that cardiac glycosides exert a positive inotropic effect not through the improvement of energy liberation, but through that of energy utilization of heart muscles. This concept has been further supported by many observations that energy utilization is impaired in the failing heart in which cardiac glycosides are most effective. Since contractile protein, i. e., Actomyosin (AM) is one of the important elements which are closely related to energy utilization of muscles, it is reasonable that some investigators have devoted particular attention to demonstrate direct

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action of cardiac glycosides on AM prepared from cardiac or/and skeletal muscles.

Aside from the problem whether the glycosides have any specific action on the failing heart, most investigators have studied on AM separated from normal skeletal or cardiac muscles. The results obtained from these studies so far have been considerably conflicting. Some workers reported that glycosides had direct action on contractile protein, such as the increase of polymerization of G-actin extracted from heart muscle, the activation or inhibition of adenosin triphosphatase (ATPase) activity, or the augmentation of contractility of glycerol-extracted muscle fibers or threads. Others, on the other hand, could not detect any effect on ATPase activity or contractility of AM models. These discrepancies might probably be due to the technical difficulties, particularly for purification of contractile protein from cardiac muscles and also to the differences of experimental conditions, such as ionic milieu, pH, temperature, and concentrations of glycosides.

The present study was undertaken, therefore, to evaluate the direct action of ouabain in various concentrations on 1) superprecipitation, 2) AM-ATPase activity, and 3) tension-development of glycerinated fibers carefully extracted from normal, both cardiac and skeletal muscles, and also from muscles of experimentally induced failing hearts.

METHODS

Psoas muscles of 5 normal adult rabbits were used for the studies on AM extracted from skeletal muscles. Actomyosin from cardiac muscles was prepared from approximately 60 normal adult dogs. Surgical avulsion of the tricuspid valve and stenosis of the pulmonary artery originally described by Barger et al.¹⁾ were performed in 6 dogs, resulting in chronic right-sided heart failure which was demonstrated by weakness, reduced tolerance to exercise, pathological findings of congestion in liver and spleen in all cases, and ascites in one case. Actomyosin solution from skeletal muscles was extracted by the original method of Szent-Györgyi, while that from cardiac muscles was extracted by modification²⁾ of the method of Kako and Bing.³⁾

Superprecipitation: Prior to the observation of superprecipitation in the water bath maintained at 20°C, AM solution was incubated for 5 minutes at 5°C after the addition of ATP solution. This modification made it possible to obtain from AM of cardiac muscles as good plugs as those from AM of skeletal muscles by the usual method for superprecipitation, i.e., the addition of ATP solution after incubation at 20°C. In the present experiments, the conditions were as follows.—pH; 7.0~8.0. Final concentrations of AM, KCl, and ATP; 1.5~2.0 mg./ml., 0.10~0.16 M, 5×10^{-4} M, respectively.

ATPase activity was determined by means of the measurement of inorganic phosphate (Fiske & Subbarow⁴⁾) liberated during 5 minutes after the addition

of ATP. The value thus obtained was about 50γ for skeletal, and 25γ for cardiac muscles, under the following conditions: The final concentrations of AM, KCl, and ATP; 2 mg./ml. 0.1 M, and 2×10^{-3} M, respectively. pH; 8.0. Temperature; 25°C.

Isometric tension: Strips of psoas muscles and trabecular muscles of both left and right ventricles were stored in 50% glycerol at 0°C for 3 days, then transferred to fresh 50% glycerol, and preserved at -20°C for at least 2 weeks. Under microscopic examination, the glycerol-extracted strips were separated into single fibers with diameter of 0.03 mm. for skeletal muscle and small fiber bundles with diameter of about 0.2 mm. for cardiac muscles. This diameter of cardiac fibers was demonstrated as the most optimal one for tension-development. Despite of greater values of tension in some experiments, finer fibers were not suitable, because there was large individual variation due to the technical difficulty. The length of both skeletal and cardiac fibers was about 20 mm. Isometric tension was measured by the modified tension-meter originally described by Weber.⁵⁾ The experiments were carried out in the medium containing 0.06 M KCl and tris-(hydroxymethyl)-aminomethane buffer (pH 8.0). The temperature was maintained at 20°C and the final concentration of ATP was 2×10^{-5} M.

All of superprecipitation, ATPase and isometric tension were observed both in the absence and the presence of Ca or Mg (10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} M). The cardiac glycoside used in the present study was ouabain, its final concentration being 10^{-7} , 10^{-6} , 10^{-5} and 2×10^{-5} M, respectively.

RESULTS

(A) AM extracted from cardiac and skeletal muscles of normal animals

(1) Effect of ouabain on superprecipitation

(a) Ca- and Mg-free medium: Lowest concentration of ouabain (10^{-7} M) accelerated superprecipitation moderately in some experiments, and slightly in other experiments. Medium concentration (10^{-6} M) unaffected in most experiments, and slightly accelerated in other experiments. On the contrary, high concentration (10^{-5} , 2×10^{-5} M) resulted in definitive inhibition. Fig. 1 shows one of these experiments.

(b) Ca- and Mg-containing medium: These actions of ouabain in both low and high concentrations observed in experiments (a) were not fundamentally altered by the addition of various concentrations ($10^{-2} \sim 10^{-5}$ M) of Ca or Mg. These results ((a) and (b)) were similarly obtained in AM solution prepared from skeletal muscles.

(2) Effect of ouabain on ATPase activity (Fig. 2)

(a) Ca- and Mg-free medium: ATPase activity of AM from cardiac muscles was unaffected by lower concentrations (10^{-6} and 10^{-7} M) of ouabain. Higher concentrations (10^{-5} or 2×10^{-5} M), in contrast, inhibited

Fig. 1. The effect of ouabain in various concentrations on superprecipitation of actomyosin extracted from cardiac muscles. The formation of plug is accelerated by lower concentrations, and inhibited by higher concentrations of ouabain. Experimental conditions: AM.; 2 mg./ml. KCl.; 0.12 M. ATP.; 5×10^{-4} M. pH.; 8.0. Temp.; 20°C. C: Control.

Fig. 2. The effect of ouabain on actomyosin ATPase activity. (a) ▨ both Mg and Ca free medium. Activity is unaffected by lower concentrations, and inhibited by higher concentrations of ouabain. (b) □ Mg containing medium (MgCl_2 : 10^{-4} M) (c) ■ Ca containing medium (CaCl_2 : 10^{-4} M). Addition of Ca or Mg does not influence on the effect of ouabain. Mean value of five experiments. Experimental conditions: AM.; 2 mg./ml. KCl.; 0.10 M. ATP.; 2×10^{-3} M. pH.; 8.0. Temp.; 25°C.

the activity. The inhibitory effect of 2×10^{-5} M was more remarkable than that of 10^{-5} M, and the activity was decreased by as much as 60%.

(b) The presence of Ca and Mg (10^{-3} M) did not influence on this

ouabain-action. All of these results were also observed in the experiments of skeletal muscles.

(3) Effect of ouabain on isometric tension of glycerol-extracted fibers

(a) Ca- and Mg-free medium: Effect of various concentrations of

Fig. 3. The effect of ouabain on maximal tension of glycerinated heart muscle fibers of both ventricles. Tension-development is inhibited by high concentration (2×10^{-5} M), and unaffected by lower concentrations of ouabain. No difference is observed between left and right ventricles. ● left ventricle. ○ right ventricle. Experimental conditions: pH.; 8.0. KCl; 0.06 M. ATP; 5×10^{-3} M. Temp.; 20°C. No Mg and Ca added.

ouabain on tension-development was similar to that on ATPase, i. e., no effect by lower concentrations ($10^{-7} \sim 10^{-5}$ M) and inhibitory effect by high concentration (2×10^{-5} M) (Fig. 3).

(b) Ca containing medium: Maximal tension-development was

Table I. The Effect of Ouabain in Various Concentrations on Maximal Tension of Glycerinated Cardiac Fibers in Ca Free and Containing Medium

Ouabain	Ca	(-)	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
(-)		100%	99%	92%	73%	45%
10^{-7} M		98%	93%	91%	91%	73%
10^{-5} M		97%	84%	87%	83%	60%
2×10^{-5} N		69%	64%	63%	50%	36%

The value in each column presents percentage of control value of maximal tension. The inhibitory effect of Ca is manifest by raising its concentration. This effect is counteracted by low concentration (10^{-7} M), and intensified by high concentration (2×10^{-5} M) of ouabain. Mean value of 5~10 experiments. Experimental conditions: pH.; 8.0. KCl; 0.06 M. ATP; 5×10^{-3} M. Temp.; 20°C. No Mg added.

diminished slightly by 10^{-5} M, moderately by $10^{-4} \sim 10^{-3}$ M and markedly by 10^{-2} M of Ca. Lower concentrations of ouabain ($10^{-7} \sim 10^{-5}$ M) counteracted this inhibitory effect of Ca. Maximal tension, however, did never exceed the control level. Thus we could not find any increase of contractility of ouabain in both Ca-free and Ca-containing medium. High concentration (2×10^{-5} M) of ouabain, on the contrary, significantly

Fig. 4. The effect of ouabain on time-course of tension-development of glycerinated skeletal muscle fibers in Ca containing medium. Inhibitory effect of Ca (10^{-3} M) on tension-development is counteracted by low concentration (10^{-7} M), and intensified by high concentration (2×10^{-5} M) of ouabain. The same experimental conditions as shown in Fig. 3.

augmented the inhibitory effect of Ca. Table I shows the combination-effects of ouabain and Ca in various concentrations. The similar combined effect of 2 substances was also observed in the tension-development of glycerinated skeletal fibers (Fig. 4).

(c) Mg containing medium: Mg augmented tension in contrast with

Fig. 5. The effect of ouabain on time-course of tension-development of glycerinated cardiac muscle fibers in Mg containing medium. The effect of ouabain is not modified by the presence of Mg. The same experimental conditions as shown in Fig. 3.

Ca. As shown in Fig. 5, the effect of ouabain in the medium containing Mg was the same with that observed in Ca- and Mg-free medium, i. e., no effect in low concentration (10^{-7} M) and inhibitory effect in high concentration (2×10^{-5} M).

(B) AM extracted from cardiac muscles of dogs with right-sided heart failure (Fig. 6)

Diminution of superprecipitation and ATPase activity was demonstrated in AM extracted from the failing right ventricles compared with

Fig. 6. The effect of low concentration (10^{-7} M) of ouabain on (A) superprecipitation, (B) ATPase activity, and (C) maximal tension of AM extracted from both right failing and left normal ventricles. Superprecipitation and ATPase activity are diminished in right failing ventricle, and tension-development is diminished in both ventricles. The effect of ouabain is similarly observed in AM extracted from both ventricles. C: Control. O: Ouabain. Ca: CaCl_2 .

that from the normal left ventricles, while tension-development was diminished in glycerinated fibers from both ventricles. Ouabain of low concentration (10^{-7} M) a) accelerated superprecipitation, and b) unaffected ATPase activity to the same degree in AM from both ventricles. c) Effect on isometric tension in the presence or absence of Ca was also observed in the similar manner with that of glycerinated fibers from normal hearts. Thus the particular improvement by ouabain was not observed on the impaired properties of AM extracted from failing hearts.

DISCUSSION

The results obtained from the present experiments could be summarized as follows: 1) Higher concentration (toxic dose) of ouabain depressed all of the properties of AM observed, i. e., superprecipitation, AM-ATPase, and tension-development of glycerinated fibers. 2) Lower

concentrations (therapeutic dose) of ouabain accelerated superprecipitation, unaffected ATPase, and counteracted the inhibitory effect of Ca on tension-development. 3) The similar results were obtained on AM extracted from all of cardiac, both normal and failing, and skeletal muscles.

The lack of the study on the effect of cardiac glycosides on superprecipitation in the past is probably due to the reason that the method to observe superprecipitation is so far semi-quantitative and also that it is difficult to obtain the compact plugs from AM solution extracted from cardiac muscles by the usual method. The accelerating effect of lower concentrations of ouabain could be observed, using the method modified by the authors. This effect, however, might not be interpreted as the action-mechanism of glycosides. Because the contractility of glycerinated fibers was not increased by ouabain though the experimental conditions were exactly not the same. Furthermore, whether this effect was specific for ouabain was not investigated in this study. As to ATPase activity, cardiac glycosides have been reported to inhibit,⁶⁾⁻⁸⁾ augment,⁹⁾ or unaffected^{10), 11)} it in the preparations extracted from cardiac or skeletal muscles. These conflicting results are probably explained by the facts that the considerations to the concentration of glycosides were insufficient and that native and unpurified muscle extracts or homogenates were used in most of the previous studies. More recent study by Read & Kelsey¹²⁾ presented non-effect of digitoxin on purified Myosin B (AM) in the absence of myokinase. In agreement with their results, no effect on AM-ATPase was observed by low concentrations of ouabain in the present study. High concentrations, on the other hand, showed a significant inhibitory effect, which might reflect the inhibition of superprecipitation and also of tension-development of glycerinated fibers.

The effect of glycosides on the contractility of AM models have been observed by using AM-threads, -bands, or glycerinated fibers. The results in these studies have been as conflicting as those in the studies on ATPase mentioned above. Some investigators reported no effect, and others an increase of contractility. Bing and his co-workers observed no effect of glycosides on the contractility of glycerinated heart muscles in the earlier study (1954).¹³⁾ In their later report (1958),³⁾ however, positive effect on AM bands was demonstrated by the addition of Ca (10^{-3} M) into medium. This positive effect in the presence of Ca was observed previously by Edman (1953),¹⁴⁾ but not by Korey (1950).¹⁵⁾ In Ca-free medium, on the other hand, Maruyama¹⁶⁾ and Fujita¹⁷⁾ reported no effect on glycerinated fibers, while Bowen¹⁸⁾ and Robb & Mallov¹⁹⁾ observed the stimulating effect on AM threads. In the present study, careful experiments were repeatedly performed as to the effect of ouabain (10^{-7} ~

2×10^{-5} M) in both Ca-free and various concentrations of Ca-containing medium. As described above, low concentrations had no effect on tension-development in the Ca-free medium. In the presence of Ca, ouabain did not improve tension-development over the control level, though it counteracted the inhibitory effect of Ca. These results do not agree with others reported by Edman¹⁴⁾ and Kako & Bing.³⁾ According to Edman, the contractility of glycerinated fibers from psoas muscles was increased by ouabain with concentrations ranging from 10^{-4} to 10^{-6} M in the presence of low concentrations (10^{-3} and 10^{-4} M), but not higher concentrations of Ca. The lower concentrations of Ca, in his experiments, did unaffected the contractility without ouabain. In our experiments, however, Ca diminished tension-development slightly in concentration of 10^{-4} M, and moderately in concentration of 10^{-3} M. Another point which was completely different from our results, is that high concentration of ouabain (10^{-4} M) did not decrease, but increased the contractility in his experiments. Recently Lee²⁰⁾ described that heart muscle fibers extracted in glycerol with 0.1 M histidine buffer for a short duration (approximately 3 to 15 days) showed an increase of contraction by ouabain without Ca ion, whereas this ouabain-effect could not be demonstrated in the fibers extracted without histidine buffer and/or for a prolonged time. He also described that "relaxing factor" was maintained in ouabain-sensitive fibers and lost in ouabain-refractory fibers. Thus he concluded that the presence of "relaxing factor" was necessary to demonstrate the effect of glycosides on AM preparations. Although his study is interesting, the problem whether glycosides have stimulating effect on AM contractile system still remains unsolved, because other workers who found the stimulating effect, did not perform their experiments with similarly glycerinated fibers in which Lee's "relaxing factor" was maintained. Furthermore, it is reasonable to suppose that AM threads or bands used by other investigators lost "relaxing factor" during the process of preparation.

As far as we know, apparently no investigators have given the particular attention to the effect of high concentrations of glycosides on contractile protein. The enhancement of toxicity of cardiac glycosides by excess of Ca has been observed in isolated hearts and also in intact animals. Recent studies demonstrated that exclusion of traces of Ca by EDTA eliminated detectable effect of toxic dose of glycosides on heart muscles.²¹⁾ Based on these findings, chelation of calcium has been reported to be useful in the therapy of digitalis intoxication.^{22), 23)} The present study showed an additive inhibitory effect of ouabain and Ca in high concentrations of both substances. This finding may indicate that membrane-free contractile protein is at least one of the locuses where

this synergism between Ca_i and glycosides occurred.

In view of the results from our experiments it was suggested that ouabain had some effect on glycerol-extracted fibers in the presence of Ca , but not in the presence of Mg . The mechanism of this effect cannot be explained in the present study. One possibility is that ouabain has a kind of plasticizing effect. Thus ouabain in lower concentrations counteracts the inhibitory effect of Ca which makes AM rigid. In higher concentration, on the contrary, this plasticizing action is excess resulting in further diminution of tension-development.

In most of previous studies, the effect of cardiac glycosides was investigated in AM prepared from either skeletal or normal cardiac muscles. Very few workers have presented the comparative study of both muscles, and no reports except one³⁾ have dealt with the failing heart. The present study may indicate that the action of glycosides is similar in both skeletal and cardiac muscles in regard to the contractile protein. The detailed studies in our laboratory^{2), 24), 25)} demonstrated that there were no qualitative differences of AM properties between cardiac and skeletal muscles in regard to superprecipitation, AM-ATPase and isometric tension of glycerinated fibers. This similarity of properties should account for the similarity of the response to glycosides. In experimentally induced right-sided heart failure, impaired properties of AM from failing hearts did not show any improvement by the treatment with ouabain, presenting the similar response to glycoside with AM from normal hearts. This finding has no correlation with the recent concept that glycosides stimulate both normal and failing cardiac muscle alike,²⁶⁾ but suggests that the direct action of glycosides on membrane-free contractile protein has minimal, if any, relation to the mechanism of positive inotropic action in either failing or normal heart. Finally as was speculated by Hajdu and Leonard,²¹⁾ it should be reconsidered whether a mechanism of the improvement of cardiac function by glycosides is directly related to the cause of the congestive heart failure.

SUMMARY

Actomyosin (AM) was isolated from the heart muscles of normal dogs and dogs with right-sided congestive failure, and also from posoas muscles of rabbits. Action of ouabain on superprecipitation, AM-ATPase activity, and isometric tension of glycerinated fibers was observed under various conditions, and it was concluded that 1) lower concentrations (therapeutic dose) of ouabain accelerated superprecipitation, and unaffected both ATPase activity and tension-development of glycerinated fibers (in Ca -free medium), 2) in higher concentrations (toxic dose), ouabain inhibited all

of the physicochemical properties of AM examined, 3) ouabain had some correlation with Ca in regard to tension-development of glycerinated fibers, since in low concentrations it counteracted the inhibitory effect of Ca, and in high concentrations it augmented this inhibition, and that 4) all of the properties of AM extracted from cardiac, both normal and failing, and skeletal muscles had the similar response to this drug. Thus the improvement of the impaired superprecipitation, ATPase activity and tension-development was not observed by the treatment with ouabain.

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