A BIOLOGICAL ASSAY METHOD FOR CARDIOTONIC STEROIDS*, USING FROG RECTUS ABDOMINIS MUSCLE**

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Many years ago Katagi (1) and Freund (2) reported that some cardiotonic steroids and their glycosides could induce a sustained contracture in such skeletal muscle rich in slow muscle fibres as rectus abdominis muscle or gastrocnemius muscle of the frog after a rather long latent period. However, any due attention was not paid to this finding until very recently, when the same phenomenon was re-observed in this laboratory (3). Further study of this phenomenon in this laboratory (4) revealed one and the same structural requirements for both the contracture-inducing action and the cardiotonic action of these compounds, although it was demonstrated that this contracture was of nervous origin, somehow mediated by acetylcholine.

In order to test the possibility whether this phenomenon can be used as an assay method for the cardiotonic action of the cardenolides and bufadienolides, the relative potencies of the eight representative cardiotonic steroids were determined, using the length of the latent period of these compounds to produce contracture in the frog rectus abdominis muscle as a measure of their contracture-inducing activity, and was compared with the relative potencies of these compounds to produce cardiotonic action.

METHODS

1. Experiments with frog rectus abdominis muscle

The experiments were performed from September to October using rectus abdominis muscle of female frog (*Rana nigromaculata*). The dissected muscle was suspended in a 5 ml organ bath containing Ringer's solution aerated with air and maintained at temperature of $23 \pm 0.5^\circ\text{C}$. The composition of Ringer's solution used was as follows: NaCl, 110 mM; KCl, 0.4 mM; CaCl_2 , 1.6 or 0.4 mM; NaHCO_3 , 1.2 mM; glucose, 4.3 mM. To know the exact concentration of calcium, the concentration of the stock solution was checked chemically, every time Ringer's solution was made. In the previous study the muscle was stored overnight in the refrigerator before use, but in the present study the muscles just after dissection were used, because of their higher sensitivity. The contracture was recorded isotonicly on a smoked drum.

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* By the word cardiotonic steroids are meant in this paper, the steroids of cardenolides and bufadienolides type and their glycosides.

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2. Experiments with isolated frog's heart (Straub's preparation)

Experiments were performed at room temperature in four consecutive days at the end of April. Straub's cannula was filled with about 2 ml of Ringer's solution aerated with pure O_2 , the surface of which reached the height of 5 cm above the center of the ventricle. The composition of Ringer's solution was the same as that used for the rectus abdominis muscle, the concentration of calcium being 1.6 mM. Contraction of heart was recorded on a smoked drum by isotonic lever with a load of about 1 g. To produce the hypodynamic state of the heart, the concentration of calcium was reduced to half of the normal, and the effects of the cardiotonic steroids were tested in this condition.

Compounds* used in the above two types of experiments were: digitoxigenin, digitoxin, dihydro-digitoxin, ouabagenin, ouabain, digoxigenin, digoxin and bufalin. Stock solutions of these agents were prepared, dissolving each compound in 70% ethanol in concentration of 1 mg/ml (10^{-3}). Just before use these stock solutions were diluted with Ringer's solution to a desired concentration. Administration of these agents was done by substituting the whole bathing medium with these solutions.

RESULTS

1. Contracture-inducing action of cardenolides and bufadienolides

FIG. 1. Tracing of the typical kymograph record of the contracture. L represents the latent period. Time : 1 min.

To compare the contracture-inducing activity of the cardiotonic steroids, the latency of the contracture of frog rectus abdominis muscle was chosen as a measure of the contracture-inducing activity. As is shown in Fig. 1, the contracture induced by cardiotonic steroids in this muscle usually occurred after a rather long latent period, during which there was often a gradual rise of tension**. Owing to the smooth transition from the initial gradual tension rise to a distinct contracture, it was often a little difficult to determine accurately the point of onset of contracture. In order to avoid this difficulty, the line of the initial tension rise and the tangent line of the

* The agents used in the present study came from the following sources: digitoxigenin, ouabain, digoxigenin, digoxin from Dr. M. Okada of Tokyo Biochemical Research Institute; digitoxin from Shionogi & Co. Ltd.; dihydro-digitoxin from Sandoz Ltd. and from Dr. A. Okano of Daiichi Seiyaku Co. Ltd.; ouabagenin from Dr. T. Reichstein of University of Basle; bufalin from Taisho Pharmaceutical Co. Ltd. The author is deeply indebted to these researchers, laboratories and companies for their kind supply of the materials.

** This tension rise was found to be of muscular origin, probably due to the shift in the depolarization-tension curve (5).

FIG. 2. Latent period-log concentration curve.

ensuing abrupt tension rise were both so extended as to cross each other and the crossing-point of these two lines was taken as the point of onset of contracture. The time after the administration of the compounds to the onset of contracture thus measured was regarded as the latent period of this contracture.

When the temperature and calcium concentration of the bathing medium was kept constant, the latent period of the contracture of frog rectus abdominis muscle became a function only of the concentration of the agents. In Fig. 2 is depicted the latency of contracture plotted against the log-concentration of cardiotonic steroids; the curves thus drawn represent a group of semi-hyperbola. One remarkable point of this figure lies in the fact that the portion of these curves corresponding to the latent period of 30-50 minutes have a tendency to parallel with each other, suggesting the probable usefulness of this phenomenon for the purpose of measuring the relative potencies of these compounds. The same situation was also observed with other agents tested.

To search further into this possibility, the following experiments were performed. Eight representative cardiotonic steroids were chosen as model compounds. Two or three concentrations at intervals of log 1.3, expected to produce contracture after a latent period of 30-60 minutes were chosen, and the latent period of contracture at these concentrations were determined, three times each. The results are summarized in Table 1. The results of analysis of variance are given in Table 2. The analysis of variance table indicates that the relationship between the latent period and log-concentration is adequately expressed as a linear regression line with each drug. It

TABLE 1. Drug concentration and latent period. $[Ca^{++}]_0$: concentration of calcium (mM) in Ringer's solution.The concentration ratio of high, medium and low was 1 : 1.3^{-1} : 1.3^{-2} .

	Drugs (high concentration)	$[Ca^{++}]_0$	Latent period (min)		
			high conc.	medium conc.	low conc.
I	Digitoxigenin ($5.1 \times 10^{-7}M$)	0.4	33	45	54
			34	43	50
			33	41	49
	Digitoxigenin ($1.3 \times 10^{-6}M$)	1.6	36	44	49
			32	45	52
			29	42	47
	Digitoxin ($3.4 \times 10^{-6}M$)	0.4	34	40	54
			31	50	59
			40	41	51
	Dihydro-digitoxin ($4.6 \times 10^{-6}M$)	0.4	38	40	52
			33	52	58
			31	47	60
	Ouabain ($3.6 \times 10^{-6}M$)	0.4	37	49	54
			43	47	60
			42	52	61
	Ouabagenin ($3.0 \times 10^{-6}M$)	0.4	26	40	51
			31	32	47
			27	39	52
	Bufalin ($3.1 \times 10^{-7}M$)	0.4	28	42	51
			31	39	55
			29	34	47
II	Digoxigenin ($2.3 \times 10^{-6}M$)	0.4	38	48	
			45	53	
			41	53	
	Digoxin ($9.0 \times 10^{-6}M$)	0.4	43	50	
			42	50	
			41	55	
	Digitoxin ($8.5 \times 10^{-6}M$)	1.6	41	47	
			32	44	
			34	46	
	Ouabain ($1.1 \times 10^{-5}M$)	1.6	36	44	
			41	53	
			32	42	

also supports the parallelism existing among these curves. The straight lines in Fig. 3 are drawn with the common slope calculated according to the multiple assay procedure. The potencies of cardiotonic steroids to induce contracture in the rectus abdominis muscle are calculated in term of digitoxigenin and listed in Table 3. As far as the three pairs of aglycones and glycosides used in this study are concerned, the aglycones had always a stronger contracture-inducing activity than the corresponding glycosides.

Early experiments were performed in 1.6 mM calcium Ringer's solution, but to

TABLE 2. Table of variance analysis.

Because the numbers of dose level in group I and group II were different, the term Preparation and Interaction were divided. P_1 and P_2 indicate the variations among agents within group I and group II of Table 1, and P_3 the variation between group I and group II. The term Linear is significant and the terms Quadratic and Interaction are not significant. This indicates that the relationship between latent period and log concentration in each agent may satisfactorily be expressed as a linear regression line with a common slope.

Factors	SS	Df	Ms
Preparation			
P_1	773.78	6	128.96**
P_2	189.13	3	63.04*
P_3	8.05	1	8.05
Dose			
Linear	4710.42	1	4710.42**
Quadratic	0.13	1	0.13
Interaction			
$P_1 \times L$	48.30	6	88.05
$P_2 \times L$	0.12	3	0.04
$P_3 \times L$	0.03	1	0.03
$P_1 \times Q$	36.30	6	6.05
Error	756.00	58	13.03
Total	6522.23	86	

* : Statistically significant at 5% level.

** : Statistically significant at 1% level.

FIG. 3. Regression line of each compound.

Each point in this figure is the mean of 3 determinations. The common gradient of these curves is -86.9 minutes per ten times increase in drug concentration.

TABLE 3. Potency ratio (on mole base).

The potency of digitoxigenin was taken as 1.00.

Agents	[Ca ⁺⁺] ₀	Potency ratio	Confidence interval
Digitoxigenin	0.4	1.00	—
Digitoxigenin	1.6	0.39	0.54 -0.28
Digitoxin	0.4	0.14	0.19 -0.10
Digitoxin	1.6	0.055	0.079-0.038
Dihydro-digitoxin	0.4	0.10	0.14 -0.072
Ouabagenin	0.4	0.19	0.26 -0.14
Ouabain	0.4	0.12	0.17 -0.087
Ouabain	1.6	0.042	0.060-0.029
Digoxigenin	0.4	0.18	0.26 -0.13
Digoxin	0.4	0.045	0.065-0.031
Bufalin	0.4	1.8	2.5 -1.3

TABLE 4. Effect of calcium on the potency.

Drugs	Potency in 0.4 mM [Ca ⁺⁺] ₀ Potency in 1.6 mM [Ca ⁺⁺] ₀
Digitoxigenin	2.58 (3.6-1.9)*
Digitoxin	2.51 (3.5-1.8)*
Ouabain	3.05 (4.2-2.2)*

* Confidence interval.

TABLE 5. Action on the isolated frog's heart (Straub's method).

— : no effect within five minutes,

+ : improvement in contractility within ten minutes, but no tendency to systolic arrest,

⊕ : systolic arrest.

Concentration (g/ml)	3 × 10 ⁻⁸	10 ⁻⁷	3 × 10 ⁻⁷	10 ⁻⁶	3 × 10 ⁻⁶	10 ⁻⁵
Digitoxigenin	— — —	+++	++⊕	⊕⊕		
Digitoxin			— — —	+++	⊕⊕⊕	
Dihydro-digitoxin			— — —	+++	++⊕	⊕
Ouabagenin		—	++—	⊕⊕⊕		
Ouabain			++	+++	⊕⊕⊕	
Digoxigenin		+++	+++	⊕⊕⊕		
Digoxin				— — —	+++	⊕⊕⊕

improve the sensitivity most of the experiments were carried out in 0.4 mM calcium Ringer's solution. As is shown in Fig. 2 and is confirmed statistically (Tables 2 and 4, Fig. 3), the variation of calcium concentration resulted only in a parallel shift of the dose-response curve, always resulting in the same increase or decrease in sensitivity, indicating the homogeneity of the responses at these two different concentrations of calcium. Thus, it was possible to analyze all the data together.

FIG. 4. Comparison of the relative potency in rectus abdominis muscle with that in isolated heart. Each open bar indicates the action on one heart. The left ends of the open bars represent the lowest concentration causing increase in contractile force and the right end, the concentration causing systolic arrest. Short shaded bars represent the concentration at which the contracture would occur after the latent period of 43.3 minutes (mean of total determinations).

2. Effect of the cardiotonic steroids upon the isolated heart

To see whether the relative potencies of the eight representative cardiotonic steroids determined in the above experiments agree well with those of the cardiotonic action, the cardiac action of the above compounds except bufalin were examined using the isolated frog's heart. The results are presented in Table 5 and Fig. 4. These data clearly demonstrated that the relative potencies of these seven compounds to produce cardiotonic action agreed well with those to induce contracture in rectus abdominis muscle.

DISCUSSION

According to the present data, the relative potency of the eight representative cardiotonic steroids to produce contracture in rectus abdominis muscle was in strikingly good agreement with that of these compounds to produce cardiotonic action, suggesting the probable usefulness of this phenomenon as a biological assay method for the cardiotonic action of those compounds.

As this contracture was demonstrated to be of nervous origin, somehow mediated by acetylcholine, such a close correlation as reported here might seem, at first sight, rather queer, and might be taken to be a mere coincidence. However, this agreement might probably be more than a coincidence. According to Otsuka (6), there are many similarities between the excitation-contraction coupling (E-C coupling) in muscle and the excitation-secretion coupling (E-S coupling) in nerve; the important portion of

these two coupling mechanisms may be quite similar or one and the same. Recently, evidences indicating that the site of action of cardiotonic steroids is E-C coupling have been increasing (7). These two taken into consideration, it seems fairly reasonable to suppose that the cardiotonic steroids may have some effect on E-S coupling, as a result of which acetylcholine is released from the nerve ending, resulting in a contracture of the innervated slow muscle fibres.

Thus the intimate relationship observed between the contracture-inducing action and the cardiotonic action of the cardenolides and bufadienolides could be taken to be the reflection of the intrinsic connection of these two actions. This situation may be depicted as follows:

In the present study, stronger action was always observed with aglycones, rather than with glycosides, both in the rectus abdominis muscle and in the isolated heart, in contradiction to the generally-accepted view on the relative potency of the glycosides and aglycones (8, 9), originated from the determination of the lethal doses in the whole animal, mainly in the cat. The determination of lethal doses of the cardiotonic steroids is actually the determination of the toxicity of these compounds to the heart in the whole animal, since the animals die of the ventricular fibrillation of the heart under this circumstance. It is true, then, that the lethal doses thus measured can be taken as a measure of the effects of these compounds on the heart, but they cannot be regarded as a measure of the effects on the isolated heart, since many processes which affect the action of these compounds are operating outside the heart in the whole animal; especially important among these is the degradation process in the liver. Those compounds which are liable to degradation tend to give an unreasonably high lethal dose, even if they have a strong action upon the heart *per se*. Thus, resibufogenin, considered to be inactive based on the toxicity data (10), have recently been found to be cardiotonic (11). According to Repke, aglycones are usually more easily transformed than the corresponding glycosides are. If so, the weak activity usually ascribed to aglycones resulted not from a weak action of aglycones on the heart, but from faster transformation rate. Rothlin (12) demonstrated that, if the higher infusion rate is adopted, the toxicity of aglycones increases. Presumably, then, at a certain high infusion rate, the toxicity of the aglycones may surpass that of the glycosides. This may be

the situation the author observed with the isolated heart.

It is no doubt that the toxicity determinations carried out by Chen, played an important role in advancing our present knowledge about the structure-activity relationship of the cardiotonic steroids, but if the true activity ratio in the heart is to be determined, it is apparent that we should study the direct action on the heart, more precisely, the inotropic action, since there are compounds like dihydro-ouabain and dihydro-digitoxin, which have a fairly strong inotropic action, but have very low toxicity ((14). The problem is the difficulty to get the quantitative evaluation of the inotropic activity. The good agreement found in the present study between the potency ratio of the contracture-inducing activity and that of the cardiotonic action and the possible intrinsic correlation between these two actions of the cardiotonic steroids as discussed above seems to warrant further examination of the rectus abdominis method, which might be a very useful tool as an assay method for the direct cardiac action of these compounds. Simplicity of the procedure of this method and the fact that only a very minute amount of the compounds is needed in this method may add to its usefulness.

SUMMARY

Relative potencies of 8 cardiotonic steroids to induce contracture in frog rectus abdominis muscle were determined using the latent period of this contracture as a measure of this action. These values were compared with those of the cardiotonic action of the same compounds in the isolated frog's heart. A good agreement of the relative potency of these two actions was observed, indicating the possible usefulness of this phenomenon as a biological assay method of cardiotonic steroids.

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