DIFFERENCE BETWEEN MECHANISMS OF ACTION OF β-ACETYLDIGOXIN, STROPHANTHIN K, AND OUABAIN

N. V. Karsanov, G. V. Sukoyan, D. R. Tatulashvili, V. N. Karsanov, and L. D. Mamulashvili

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This investigation forms part of a cycle of research aimed at explaining the molecular mechanism of action of various cardiac glycosides [2-4]. It is devoted to the definition of the effects of Ca^{2+} and glycosides and to the clarification of certain molecular mechanisms of action of β-acetyldigoxin, strophanthin K, and ouabain on the system of contractile proteins of the cardiomyocyte and, in particular, on its Ca-sensitivity and transformation of energy.

EXPERIMENTAL METHOD

Experiments were carried out on 20 normal chinchilla rabbits weighing 2-4 kg. The animals were killed under hexobarbital anesthesia.

Skinned muscle fibers (SMF), the isolated intact contractile apparatus of the cardiomyocytes, were obtained by the method in [6, 11].

The contractile properties of SMF during isometric contraction were investigated on a new generation strain gauge system, in which a 6M × 1B mechanotron was used as the sensitive element.

The SMF were fixed in the apparatus in the vertical position, by one end to A stationary rod, by the other, by means of a band, to the rod of the mechanotron, and then immersed (by lifting the cuvette) in medium I – a solution containing inhibitors of endogenous proteases and mitochondrial ATPases, as well as 0.1 M KCl, a protector of SH-groups, 2 mM $MgCl_2$, 4 mM EGTA, 1 mM dithiothreitol, 5 mM NaN_3, 0.5 mM phenylmethylsulfonyl fluoride, and 15 mM imidazole, ionic strength 0.125, pH 7.4, pCa > 8, and stretched with a force of 0.2 mN. The medium I was then replaced for 10 min with medium II, a relaxing solution (medium I + 5 mM ATP) and SMF were contracted by replacing the relaxing medium by contracting solution (medium II + $CaCl_2$).

The time taken for tension to flatten out on a plateau (both in the control and under the influence of glycosides) was under 1 min.

The coefficient of cooperativeness of the Ca-response (n) of SMF was determined by Hill's equation:

$$lg \frac{P/P_0}{1-P/P_0} = n\ lg\ [Ca^{2+}] - lg\ K,$$

where P_0 denotes the maximal tension developed at saturating Ca^{2+} concentrations, P the tension at a concrete value of pCa, and K a constant equal to pCa necessary to achieve half the maximal tension.

Binding of calcium by SMF was determined by a radioisotope method using ^{45}Ca and ^{3}H-glucose [8].

ATPase activity of SMF was determined [10] at the time of completion of the period of generation of active force (at the end of 1 min) and in the period of maintenance of a contracted state (at the end of the experiment, 5 min after the beginning of contraction). To convert the released quantity of inorganic phosphate into an equivalent change of free energy

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TABLE 1. Changes in Free Energy of ATP Hydrolysis, Generated Force, and Number and Life Span of AMESB during Contraction of SMF of Normal Heart and under the Influence of Glycosides (M ± m)

Period	Parameter	Control	β-acetyl-digoxin	Strophan-thin K	Ouabain
Complete	P_{max}, mN/mm^2	28,5±5,6	38,9±1,7*	46,8±5,6*	29,1±7,8
	ΔG_{max}, mJ/mg	70,9±3,8	110,2±1,7*	106,7±2,2*	71,8±5,1
	P/ΔG, mN/mJ	0,41±0,03	0,37±0,02*	0,54±0,05*	0,41±0,06
	t of generation of $\frac{1}{2}$ F_{max}, sec				
	t of flattering out on plateau, sec	22,1±2,2	17,5±1,3	17,1±1,5	23,5±3,5
		7	6	6	5
Generation of force	Average velocity P, mN/mm^2·min	57,6±2,2	45,5±5,6	57,6±2,4	59,2±1,9
	ΔG, mJ/mg·min	67,3±4,5	77,4±4,4*	101,4±26,9*	62,1±4,4
	Number of AMESB, μmole/mg	39,1±3,0	45,3±3,6	47,6±5,4	41,6±4,0
	P/mole AMESB, mN/mm^2·min	76,9±2,3	67,0±4,7	95,5±1,5	68,2±5,6
	ΔG/mole AMESB, mJ/mg·min	0,87±0,05	1,25±0,08*	1,07±0,10*	0,88±0,09
	P/ΔG, mN/mJ	0,54±0,03	0,73±0,08*	0,49±0,06	0,51±0,06
	t of life of AMESB, msec	1,80±0,13	1,90±0,10	2,22±0,23*	1,67±0,11
		5,8±0,2	4,9±0,4*	6,7±1,1	5,6±0,4
		n 13	13	12	5
Maintenance of contracted state	P, mN/mm^2	28,5±5,6	38,9±1,7*	46,8±5,6*	29,1±7,8
	ΔG, mJ/mg·min	8,0±0,4	17,0±0,6*	14,8±0,4*	7,6±0,6
	Number of AMESB, pmoles/mg	95,0±9,3	54,9±3,5*	86,0±4,5	95,6±9,1
	P/mole AMESB, mN/mm^2	0,30±0,07	0,69±0,07*	0,58±0,09*	0,31±0,08
	ΔG/mole AMESB, mJ/mg·min	0,080±0,003	0,31±0,06*	0,17±0,02*	0,080±0,004
	P/ΔG, mN/mJ	3,75±0,2	2,2±0,2*	3,4±0,2	4,1±0,8
	t of life of AMESB, msec	44,0±2,0	7,3±1,4*	17,0±3,0*	43,0±3,0
	t of proteolysis to $\frac{1}{2}$ F, sec	5,4±0,2	2,6±0,3*	8,9±0,9*	5,8±0,7
		n 7	6	6	5

Legend. When the number of AMESB was calculated it was assumed that 1 mg of heart muscle contained 82 pmoles myosin [12]. Asterisk indicates data for which $p < 0.05$ compared with normal.

(ΔG) of ATP hydrolysis, it was assumed that the change in the free energy of ATP hydrolysis (energy used in generating the active force and performing work) is 57 kJ/mole [9].

The number of actomyosin ensembles formed by a strong bond (AMESB) was determined by the method of restricted proteolysis of SMF (myocardial myofibrils) by trypsin [5] at the moment the tension flattens out on plateau after 5 min of contraction. During generation of force, the transition of the AME formed by a weak bond into the conformation of strong binding was inhibited by the addition of 0.3 mM sodium vanadate [1]. The rate of proteolysis during the period of maintenance of tension was determined from the time taken for the tension to fall by half.

To study the effect of glycosides on the development of force, the SMF were incubated before the experiment for 40 min at 25°C in medium I containing either 10^{-6} M strophanthin K or 10^{-6} M β-acetyldigoxin, or 10^{-6} M ouabain and the course of the control experiment was repeated, during which the SMF was washed to remove unbound glycoside at times when the fibers were in different media.

According to the results of electrophoresis, SMF contain all protein fractions of myofibrils.

Proof of the absence of functionally active mitochondrial membranes and membranes of the longitudinal sarcoplasmic reticulum and sarcolemma was obtained, but absence of an effect of 10 mM caffeine on the tension developed by SMF in medium with 1 mM Ca-EGTA buffer (unable in this concentration to bind all the Ca^{2+} discarded by the triads [7]) indicates absence of any significant number of triads capable of releasing Ca^{2+} in SMF.

Methods of statistical analysis of the results and also of determination of the free Ca^{2+} concentration were given in [5].

EXPERIMENTAL RESULTS

Replacement of the relaxing medium by contracting converts SMF from the state of rest into the state of forced generation (starting with pCa 7.2), combined with a corresponding increase in the rate of ATP hydrolysis, the maximum of which is reached at pCa 6. A further increase in the Ca concentration in the medium to pCa 4 results in no marked

increase of tension in ΔG. However, if SMF are preincubated with 10^{-6} M β-acetyldigoxin or 10^{-6} M strophanthin K, an additional increase of tension (by 1.4 and 1.6 times in the case of β-acetyldigoxin and strophanthin K respectively), above the level of purely Ca-dependent activation of the contractile process (Table 1), and an additional increase in Ca, Mg-ATPase activity or, by calculation, an increase of 1.5 times in ΔG of ATP hydrolysis occurs. In this case the effects reach their peaks at a lower Ca^{2+} concentration in the medium, and just as in the control, after the tension versus Ca,Mg-ATPase curve has flattened out on a plateau, a further decrease of pCa gives no additional rise of tension and ATPase (as is also the case with auxotonic, near-isometric contraction of myocardial fibers glycerinized for one month [2, 3]). It was found under these circumstances that the quantity of Ca^{2+} bound in the presence of glycosides was unchanged compared with the control.

Consequently, the significant growth of additional tension and ΔG of ATP hydrolysis of SMF under the influence of strophanthin K and β-acetyldigoxin over the maximal level are not mediated indirectly through Ca^{2+}, but are the result of the direct action of the two cardiac glycosides on the system of myocardial contractile proteins.

The number of AMESB (Table 1) at the end of the period of forced generation in the case of β-acetyldigoxin was found to be equal to the control, but with strophanthin K it was significantly increased. In the period of maintenance of the contracted state, however, the number of AMESB in the case of strophanthin K remained at the normal level, whereas with β-acetyldigoxin it was reduced by 1.7 times ($p < 0.01$). The decrease in the number of AMESB in this period when β-acetyldigoxin was used was accompanied by an increase in the rate of proteolysis of myosin in AMESB by half compared with the control ($p < 0.001$), but in the case of strophanthin K, just as in the period of maintenance of the contracted state, it was 1.6 times slower ($p < 0.001$). This means that in the case of interaction of SMF with β-acetyldigoxin and strophanthin K in the period of maintenance of the contracted state AMESB acquires conformations that differ from each other and from normal: in the case of β-acetyldigoxin it is characterized by greater accessibility of myosin to the action of trypsin, and in the case of strophanthin K, its greater degree of protection than normally. In the period of forced generation, we were unable to determine the conformational state of AMESB during interaction with glycosides by the technique used, but without any argument to the contrary, we may assume that for each glycoside it is the same as in the period of maintenance of the contracted state. This assumption was supported also by calculation of the life span of AMESB (which is evidently linked with its conformation), which shows that the life span of AMESB in the case of β-acetyldigoxin, just as during periods of maintenance of the contracted state and force generation, decreases (the AMESB cycle is accelerated), whereas in the case strophanthin K it is lengthened (the cycle is slowed). Since in periods of force generation and maintenance of the contracted state a single AMESB generates approximately the same tension under the influence of both glycosides, and at approximately equal rates, it can be concluded that differences in the conformation of AMESB observed in the case of β-acetyldigoxin and strophanthin K have no appreciable effect on the magnitude of the force generated by AMESB.

The conformational state of AMESB evidently determines the economy of the contractile process: in the case of strophanthin K economy is either increased (in the period of force generation) or remains at the control level (in the period of maintenance of contraction) whereas in the case of β-acetyldigoxin it remains either at the control level (in the period of force generation) or below it (in the period of maintenance of contraction). As a result, according to the data for the complete period of contraction of SMF, economy of energy utilization in the case of β-acetyldigoxin has a tendency to fall, whereas in the case of strophanthin K it increases. This is in agreement with previous findings in direct calorimetric studies on myocardial fibers glycinerized in the course of a month [3, 4], which indicate that β-acetyldigoxin has a mainly quantitative action of energy transformation, whereas strophanthin K has both quantitative and qualitative actions.

The high degree of economy of the force generation process in the case of strophanthin K is thus linked with a more compact conformation of AMESB, which has a longer life span, whereas the tendency for economy to decrease in the case of β-acetyldigoxin is connected with a more open conformation, with a shortened life span. Lengthening of the life span of AMESB evidently guarantees more complete performance of the conformational changes in AMESB which lie at the basis of force generation, whereas shortening, on the other hand, leads to their incompleteness and, consequently, to the wastefulness of the contractile process.

The fact will be noted that during transition from the period of force generation to the period of maintenance of the contracted state the AMESB cycle is slowed in the presence of the glycoside by a much lesser degree than normally, and for that reason the life span of AMESB is lengthened by a lesser degree (by 1.4 and 2.5 times in the case of β-acetyldigoxin and strophanthin K respectively, although differences in the action of β-acetyldigoxin and strophanthin K are

preserved) than normally (by 7.6 times). This is combined with a tendency (reaching the level of significance in the period of maintenance of the contracted state of SMF) to a decrease in the number of AMESB in the case of β-acetyldigoxin and an increase in their number in the case of strophanthin K in the period of force generation.

The increase in tension developed by SMF under the influence of the two glycosides above the maximal tension developed as a result of Ca-dependent activation of the contractile process, can evidently be reduced to an increase in Ca-sensitivity under the influence of the two glycosides (the force generation curve is shifted to the left by 0.4 pCa unit) and to an increase in cooperativeness of the contractile process of SMF (an increase of 1.64 times), which is based on an increase in cooperativeness of the response of the thin filament to binding of Ca^{2+}: it responds with the transition of a much larger number of functional units than normally into the activated state.

Another difference in the molecular mechanism of action of β-acetyldigoxin and strophanthin K is associated with the response of AMESB to removal of free Ca^{2+} from the medium, it being taken from troponin C: in the case of β-acetyldigoxin relaxation develops (destruction of the strong bond in AMESB and its dissociation) at the same rate as normally, but in the case of strophanthin K, relaxation is abruptly slowed. This may be evidence of a change in strength of the bond between troponin C and Ca^{2+} or, more probably, the strength of the bond between actin and myosin in AMESB, and it may explain the marked ability of strophanthin K to induce a state of myocardial contracture.

Finally, our results are evidence that the mechanism of action of ouabain (strophanthin G) is not identical with the mechanism of action of strophanthin K and of β-acetyldigoxin on SMF in vitro. Under the influence of ouabain, the magnitude and velocity of the generation tension and also the magnitude and velocity of the change in free energy of ATP hydrolysis by SMF, the quantity of Ca^{2+} bound by SMF (1.24 ± 0.07 nmoles/mg), the life span and number of AMESB in the phases of force generation and maintenance of the contracted state, and also the velocity of relaxation of SMF as a result of removal of free Ca^{2+} and the velocity of proteolysis of myosin in SMF by trypsin are unchanged. This is further conformation of the view that ouabain, like rhodeside (a cardiac glycoside obtained from *Rhodea japonica*), changes the magnitude of the force generated by the system of contractile proteins only if an intact system of the sarcoplasmic reticulum is present (in SMF obtained by the method described above they are inactivated); the triad, as was shown in 1984 [2], acts indirectly through the more rapid release of Ca^{2+}. This conclusion is in agreement with the discovery of the ouabain binding center in the triads.

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