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Original Contributions

Contractile proteins in globally "stunned" rabbit myocardium

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Summary: The isolated working rabbit heart preparation was used to study whether the "contractile machinery" remains unchanged in globally stunned myocardium. The function of the heart has been measured in nonischemic and postischemic conditions. The effect of isoprenaline or calcium chloride administration in both conditions was also studied. Myocardial contractile function was significantly depressed after 20-min global ischemia and returned to normal after $CaCl_2$ and supranormal values after isoprenaline administration. From hearts used in experiments myofibrils were prepared and their ATPase activity was determined. It was observed that myofibrils prepared from "stunned" myocardium showed about 50 % increase in ATPase activity in the presence of $CaCl_2$. Subjection of the heart to ischemia caused a decrease in calcium sensitivity of the myofibrillar ATPase. Myofibrils obtained from ischemic hearts but subjected to isoprenaline or $CaCl_2$ administration exhibited increased calcium sensitivity over that of control heart. These effects were accompanied by changes in the extent of phosphorylation of troponin I (TNI) and myosin light chains. The modification of contractile apparatus in the postischemic period described in this paper may contribute to the overall mechanism of myocardial stunning.

Key words: "stunned" myocardium; ischemia; contractile proteins; myofibrillar ATPase; phosphorylation of contractile proteins

Introduction

Reversible contractile failure after a short ischemic insult in the presence of viable myocardium tissue is a well-defined phenomenon called "myocardial stunning" (5). Since this contractile failure can be easily reversed by paired pacing or inotropic stimulation (2) and calcium infusion (13), one would expect an intact "contractile machinery" in stunned myocardium. However, it has been suggested (5) that derangement induced by ischemia in contractile proteins would be responsible for the transient postischemic left ventricular dysfunction. In the opinion of Kloner et al. (14) the inotropic stimulation of stunned myocardium should be undertaken only with great caution, due to possible residual ultrastructural and biochemical abnormalities in the tissue. Decreased actomyosin ATPase activity after a short period of ischemia in isolated rat hearts has been observed (4). Disruption of myofibrillar energy use in the postischemic myocardium as the decreased creatine kinase activity associated with the myofibril and limitation of substrate (ADP) necessary for maximal creatine kinase activity has been reported by Greenfield and Swain (8), who suggested that inotropic stimuli may overcome the functional deficit. Decrease in

the Ca^{2+} transient or decrease in myofilament Ca^{2+} sensitivity is proposed by Kusuoka et al. (15) as a pathophysiology of stunned myocardium. Studies of Schaper et al. (20) on ATP turnover provided evidence that ATP utilization in reperfused myocardium after the short period of ischemia is reduced in the presence of sufficient ATP supply. The cause of reduced ATP splitting may lie in the sarcomeres themselves. To get more insight into the mechanism of myocardial stunning, we used isolated working rabbit heart preparation (1). The state of the "contractile machinery" in the postischemic dysfunction and in the postischemic dysfunction reversed by the administration of isoprenaline or calcium chloride has been investigated by determining the myofibrillar ATPase activity, its calcium sensitivity, and the phosphorylation levels of TNI and cardiac myosin light chains.

Materials and methods

Male and female adult (New Zealand) white rabbits (1.5–2 kg) were premedicated (Hypnorm 0.4 ml/kg i.m.) anesthetized (Nembutal 25 mg/kg i.v., Pavulon 0.1 mg/kg i.v.) and artificially ventilated (Bird respirator). After heparinization (5000 U.I.) the heart was excised through left thoracotomy and put into ice cold St. Thomas' Hospital cardioplegic solution. The aorta was cannulated and the heart perfused under constant pressure (60 mm Hg). The left atrium and pulmonary artery were cannulated. Perfusion buffer-modified Krebs-Henseleit-bicarbonate solution (1) was kept at 37 °C and vigorously bubbled with a mixture of 95 % O_2 and 5 % CO_2, resulting in perfusate of pH 7.4. The heart's own rhythm was maintained. A tip catheter (Honeywell) was inserted into the left ventricle via the apex. After an equilibration period of retrograde Langendorff perfusion, the heart was exposed to constant preload (10 mm Hg) and afterload (50 mm Hg), and switched to the "working state".

Experimental protocols

The isolated hearts were divided into six experimental groups: I) Nonischemic control group. After 30 min Langendorff perfusion the hearts were exposed to the working conditions for 80 min and hemodynamic measurements were assessed every 10 min. II) Nonischemic control hearts exposed to the perfusate containing 5 mM $CaCl_2$ during the last 10 min of working state. III) Nonischemic control hearts treated with isoprenaline (5×10^{-8}). IV) Ischemic group. After 30 min Langendorff perfusion followed by 30 min working state, 20 min normothermic (37 °C) global ischemia was induced followed by the retrograde Langendorff reperfusion. After the reperfusion the system was switched to the working conditions, hemodynamic values were measured and compared to the preischemic values. V) Ischemic group treated with 5 mM $CaCl_2$ during working state after ischemia. VI) Ischemic group treated with isoprenaline (5×10^{-8} M) during the working state after ischemia.

Transmural biopsies were taken from the free wall of the left ventricle after placing the heart in the ice cold St. Thomas Hospital cardioplegic solution and cooling the heart by flushing via the aortic cannula with 50 ml of the cardioplegic solution. Biopsy specimens were immediately cooled in liquid nitrogen and kept frozen (–80 °C) until analysis.

Preparation of cardiac myofibrils

Purified cardiac myofibrils were prepared from the rest of myocardial tissue by the method of Solaro et al. (23) after biopsies were taken. The final pellet of myofibrils was resuspended to a concentration of 5 mg/ml. Occasionally, myofibril suspension was filtered through a fine nylon sieve to get better homogeneity of suspension. This procedure was found effective in improving the reproducibility of ATPase measurements. The samples of myofibril suspensions were used for determination of the extent of PLC_2 (phosphorylated myosin light chains) and phosphorylated TNI (see below).

Protein determination

50–150 µl of resuspended myofibrils were used in three parallel samples for determination of protein concentration by biuret method (7) using bovine serum albumine as standard.

ATPase assays

Myofibrillar ATPase activity was measured in medium containing 25 mM Tris-HCl buffer pH 7.0, 30 mM KCl, 2.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 2.5 mM ATP, appropriate 2 mM Ca-EGTA buffer. Myofibrils were added to the final concentration of 0.5 mg/ml. The final volume of the sample was 2 ml. Reaction was started by the addition of ATP solution, and proceeded for 5 min at 25 °C, then terminated by the addition of 1 ml 10 % (w/v) trichloroacetic acid solution. Inorganic phosphate was determined by the method of Fiske and Subbarow (6).

Ca-EGTA buffers

50 mM equimolar Ca-EGTA solution and 50 mM EGTA solution were mixed in different proportions to obtain stock solutions with required (after dilution to ATPase assay conditions) free calcium concentration, calculated using the computer program of Perrin and Sayce (18).

Determination of the extent of phosphorylation of P-light chains of myosin and troponin I

Biopsy specimens cooled in liquid nitrogen were prepared for electrophoresis as described by Westwood and Perry (26). The myofibril suspensions (1 ml) were centrifuged and the pellets were resuspended in 9 M urea or homogenized in a buffer consisting of 100 mM sodium pyrophosphate pH 8.8, 5 mM EGTA, 50 mM NaF, 10 % glycerol, 15 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride for extraction of contractile proteins (21). Before the electrophoresis the solid urea was added to the samples to the concentration of 9 M. Two-dimensional gel electrophoresis as described by Westwood and Perry (26) and, additionally, the urea-glycerol polyacrylamide gel electrophoresis (22) were performed. The extent of P-light chains was calculated on the basis of densitometric scans.

Results

In the nonischemic control hearts all hemodynamic parameters were stable during 80 min working state. Addition of isoprenaline significantly increases heart rate to 132 %, +dP/dt to 142 % and –dP/dt to 115 % of the control values. Administration of 5 mM $CaCl_2$ caused slight but not significant increase in all above-mentioned parameters in the nonischemic hearts. The function of the heart after ischemia was significantly depressed. Cardiac output decreases to 69 %, +dP/dt to 80 % and –dP/dt to 80 % of the control preischemic values. Administration of $CaCl_2$ reversed the depression of function to the preischemic value. Isoprenaline induced supranormal revovery of function after ischemia: heart rate increases to 113 % and +dP/dt to 112 % of the preischemic value.

Each heart was tested for the myofibrillar ATPase activity. The myofibrils were obtained in such a way as to assure preservation of phosphorylation levels of contractile proteins (23).

Table 1. The myofibrillar ATPase activity of myofibrils prepared from rabbit hearts, hearts subjected to ischemia, and hearts after isoprenaline or calcium chloride administration in nonischemic and post-ischemic conditions. The ATPase activities are expressed as nmol of P_i liberated per milligram of myofibrils per min. The percent of regulation was calculated according to the following formula $[(V_{Ca} - V_{EGTA})/V_{Ca}]*100\,\%$.

Myofibrils prepared from	1 mM EGTA	n	0.1 mM $CaCl_2$	n	% of Regulation
Control hearts	13.5 ± 5.3	5	70.4 ± 6.4	6	80.8
After isoprenaline administration	17.3 ± 9.2	4	85.5 ± 5.4	4	79.8
After $CaCl_2$ administration	30.3 ± 8.8	4	119.2 ± 20.1	4	74.6
"Stunned" hearts	21.2 ± 11.2	6	107.0 ± 7.9	5	80.2
After isoprenaline administration	15.1 ± 2.7	3	90.5 ± 7.5	3	83.3
After $CaCl_2$ administration	28.2 ± 7.0	4	122.3 ± 14.8	4	76.9

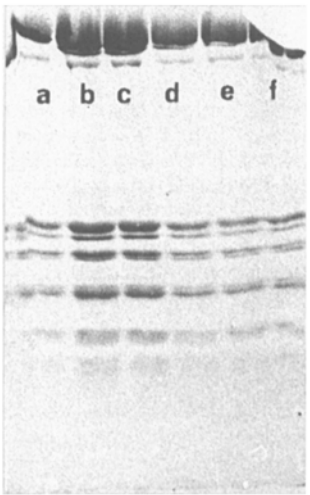

Fig. 1. SDS-polyacrylamide gradient gel electrophoresis (16) of high salt extracts from cardiac myofibrils obtained from: a) control heart, b) stunned myocardium, c) heart after calcium administration, d) stunned myocardium after calcium administration, e) heart after isoprenaline administration, and f) stunned myocardium after isoprenaline administration.

The results are presented in the Table 1. Significant differences of specific ATPase of myofibrils can be observed. The group of control hearts (not subjected to ischemia) gives values of specific ATPase activity comparable with values reported previously for rat and bovine hearts myofibrils (3, 19). All myofibril preparations exhibit calcium regulation in the normally observed extent (75–83 %). The myofibrils from ischemic hearts and ischemic and

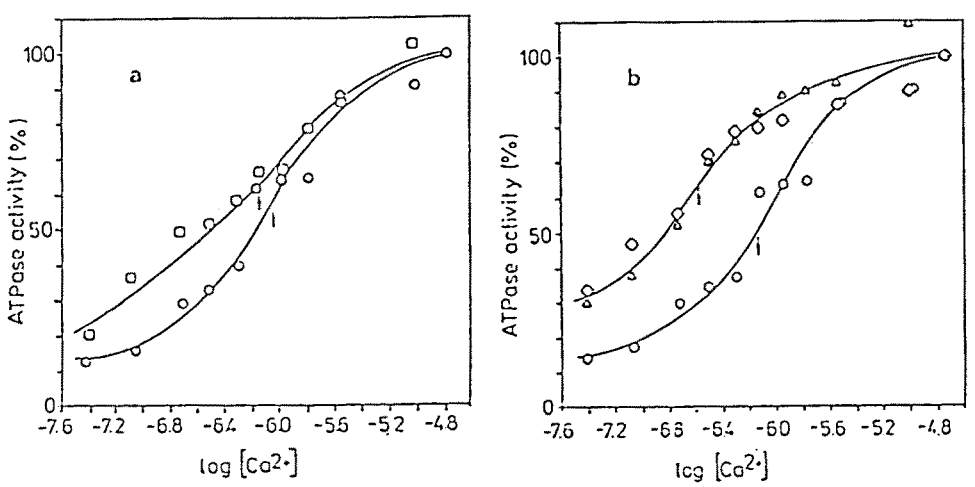

Fig. 2. Calcium dependence of myofibrillar ATPase activity. Myofibrils prepared from a: (□) control heart, (○) heart with stunned myocardium; b: (◇) heart after ischemia with administered calcium chloride, (△) heart after ischemia with administered isoprenaline, (○) heart with stunned myocardium.

nonischemic hearts subjected to administration of isoprenaline or calcium chloride show in the presence of calcium statistically significant increase of ATPase activity over control preparation (Table 1). The increase reaches the highest value (about 70 %) for hearts after $CaCl_2$ administration. In case of hearts with administered calcium chloride or isoprenaline the previous period of ischemia does not influence the ATPase activity. Myofibrillar preparations from hearts undergoing different treatment were controlled electrophoretically if they do not show differences in contractile proteins content (see Fig. 1).

Depending on the source heart, the myofibrillar preparations show different calcium sensitivity. Figure 2a represents the calcium dependence of myofibrillar ATPase activity on free calcium ions concentration of control and ischemic heart. The curve representing preparation from "stunned myocardium" reveals a shift toward a higher calcium concentration, e.g., decrease in calcium sensitivity. Administration of isoprenaline or calcium chloride after a period of ischemia causes a shift of the curve toward lower calcium concentrations (higher calcium sensitivity) in comparison to the ischemic heart (Fig. 2b). A question arises about what are the changes in the contractile apparatus responsible for the observed effects on myofibrillar ATPase activity and calcium sensitivity. To explore the possibility that phosphorylation of cardiac contractile proteins might be a cause for such behavior, the determination of TNI and myosin light chains phosphorylation has been assessed, both in biopsies and myofibrillar preparations. It has been observed that the preparation of myofibrils does not significantly change phosphorylation levels of TNI and LC_2, as compared to biopsies. In our experiments, the TNI phosphorylation was not significantly changed comparing preparations of ischemic and control heart myofibrils. A remarkable increase in phosphorylation level of TNI, however, has been observed for preparations from ischemic heart subjected to administration of calcium chloride or isoprenaline. An example

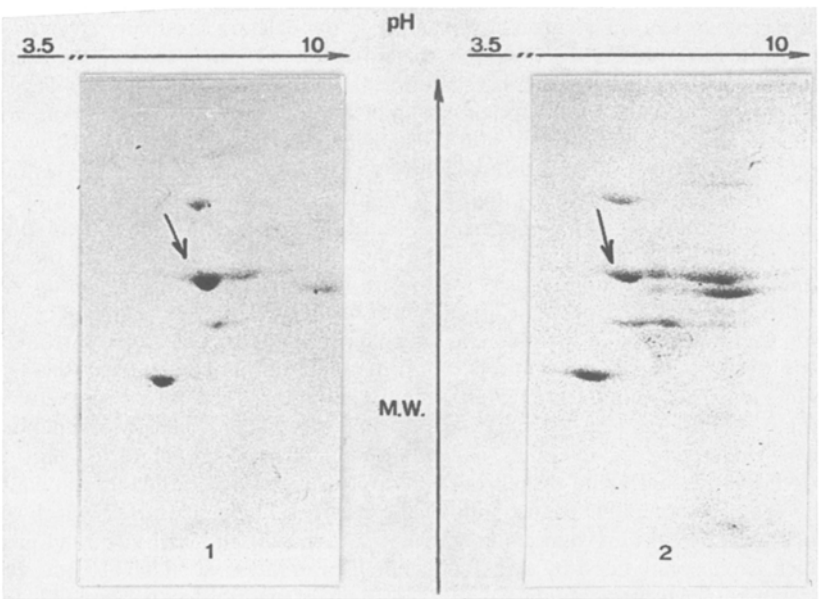

Fig. 3. Fragments of two-dimensional electrophoretic gels of biopsies taken from heart muscles subjected before working state to (1) ischemia and isoprenaline administration, and (2) ischemia. Arrows indicate the phosphorylated form of TNI.

Table 2. The changes of phosphorylation of myosin light chains of rabbit heart myofibrils in response to subjection to ischemia and reperfusion. The PLC_2 contents expressed in % relative to the total LC_2, $n \geq 3$.

Myofibrils prepared from	Relative amounts % of total LC_2	
	LC_2	PLC_2
Control hearts	57 ± 6	43 ± 6
After isoprenaline administration	41 ± 5	59 ± 5
After $CaCl_2$ administration	41 ± 2	59 ± 2
"Stunned" hearts	51 ± 6	49 ± 6
After isoprenaline administration	66 ± 4	34 ± 4
After $CaCl_2$ administration	32 ± 6	68 ± 6

of the influence of isoprenaline administration on the increase of amount of phosphorylated TNI (marked by arrows) is shown in Fig. 3. The phosphorylation levels of myosin light chains determined in myofibrils are indicated in the Table 2. The extent of phosphorylation of myosin light chains in myofibrils prepared from hearts subjected to ischemia do not differ from myofibrils obtained from control hearts. The administration of calcium chloride during the working state after reperfusion increases the level of myosin light chains phosphorylation, whereas, we observed a reversed effect after administration of isoprenaline.

Discussion

The reversible myocardial dysfunction could be connected with temporal modification of contractile proteins causing changes for example in the calcium sensitivity of actin-myosin interaction. In our studies, we observed that in "stunned" myocardium the contractile apparatus has modified properties. The myofibrillar ATPase activity at maximal $[Ca^{2+}]$ is elevated and the calcium sensitivity of the ATPase is reduced. The question arises of whether these observations correlate with the deficiency in cardiac function. The increase in myofibrillar ATPase does not seem to straightforwardly influence the functional parameters of heart contraction. The administration of calcium chloride or isoprenaline in the postischemic period restores the function of heart, but the ATPase activity still remains elevated, like in nonischemic preparations treated in the same way. The shift of the calcium dependence curve for myofibrillar ATPase toward higher calcium concentrations observed by us and interpreted as a decrease in calcium sensitivity is in good agreement with the suggestion that a decrease in myofilament calcium sensitivitiy is one of the factors causing contractile dysfunction (15). Additional confirmation for this comes from the fact that isoprenaline and $CaCl_2$ administered after ischemia are able to increase calcium sensitivity of myofibrillar ATPase and to restore, at the same time, the contractile function. The mechanism underlying these effects is not known, but a potential factor may be the phosphorylation of contractile proteins. There are at least three targets in the contractile apparatus that are susceptible to such modifications: myosin light chains LC_2 which undergo phosphorylation by calcium calmodulin activated kinase (25) and TNI (24), and protein C (10), which is phosphorylated by cAMP-dependent protein kinase. The TNI phosphorylation has been known to decrease the calcium sensitivity of myofibrillar ATPase (12, 19). The role of cardiac myosin light chains' phosphorylation is not well known, but some studies suggest that phosphorylation/dephosphorylation of the myosin P-light chains is involved in the modulation of cardiac contractility and calcium sensitivity (17). The phosphorylation of

C-protein decreases the time of twitch relaxation (9) and was found to decrease actomyosin ATPase activity in vitro (11). The concerted action of all these processes may account for the changed behavior of contractile apparatus in "stunned" myocardium: however, we were not able to show a direct correlation between the two phosphorylation levels (TNI and myosin light chains) monitored by us, and the observed effects on myofibrillar ATPase.

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