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Effect of low calcium on high-energy phosphates and sarcolemmal Na^+/K^+-ATPase in the infarcted-reperfused heart

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This study was undertaken to compare the effect of low to normal serum calcium on biochemical parameters in the myocardium of dogs subjected to 90 min of coronary artery ligation followed by 30 min reperfusion. The accumulation of calcium, the decrease of adenosine triphosphate (ATP) and creatine phosphate (CP) and the inhibition of sarcolemmal ouabain-sensitive Na^+/K^+-ATPase which are prominent findings in the ischemic-reperfused myocardium, were studied under normal and low serum Ca produced by normal and modified hemodialysis (HD). The results showed a lower accumulation of Ca ($P < 0.002$) in the ligated-reperfused myocardium of dogs subjected to low-calcium HD. In the same group of animals ATP was protected to some extent while CP was completely preserved. This may indicate that during reperfusion with low Ca, restored ATP is further utilized for CP regeneration. The activity of Na^+/K^+-ATPase was within normal values in the ligated-reperfused myocardium of the low-calcium group. The significantly ($P < 0.001$) negative correlation between tissue calcium concentration and Na^+/K^+-ATPase activity under various conditions examined, provided additional evidence that low calcium is a protective factor of the enzyme activity during ischemia and reperfusion.

Introduction

It is a well-known fact that myocardial ischemia, produced by infarction of a coronary artery, causes dramatic alterations in heart function and metabolism [1,2]. Most of these result from the decrease in the supply of both oxygen and substrates. Since oxygen is insufficient for aerobic respiration in the ischemic tissue, ATP is produced by anaerobic glycolysis and by creatine phosphate (CP) through creatine kinase (CK) [3,4]. During prolonged ischemia the depletion of ATP and CP stores as well as the retention of products of metabolism in cells, affect ATPases that mediate the active transport of ions, such as the sarcolemmal $Na^+ + K^+$-ATPase (EC 3.6.1.3) [5–7]. Reperfusion restores the production of ATP and CP to a small extent [6,8]. However, increased intracellular influx of calcium due to perturbations of ion transport systems in the sarcolemmal membrane, aggravates the myocardial dysfunction [9–11].

In previous reports [12,13], we have shown that low serum calcium produced by HD with dialysate low in calcium prevents the changes in electrolytes content and the depletion of CK during ischemia and reperfusion. Procedures designed to limit the influx of calcium during ischemia and reperfusion, such as treatment with calcium antagonists or with perfusate low in calcium, were performed in isolated myocardial preparations [8,14–18]. This study intends to examine the effect of low extracellular calcium on high-energy phosphates (ATP and CP) and sarcolemmal Na^+/K^+-ATPase activity by means of intact animals. As regards Na^+/K^+-ATPase, it is considered that its inhibition is in part responsible for the excessive intracellular calcium entry through the Na^+/Ca^{2+} system [5–7]. However, it is not clear to what extent low tissue calcium affects the enzyme activity under these pathological conditions.

Abbreviations: CP, creatine phosphate; HD, hemodialysis.

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Materials and Methods

Studies were made on 25 dogs weighing 15–25 kg. They were anaesthetized with intravenous sodium pentobarbital 30 mg/kg. The trachea was intubated and ventilation was maintained with room air using respiratory pump. A catheter was inserted via the left femoral artery to the aorta and the aortic pressure was measured with pressure transducer. Pressure signals and electrocardiogram were recorded on a 12-channel recorder. During the last 30 min of the experiments the catheter was advanced to the left ventricle (LV) to determine the LV pressure and the first derivative of LV pressure (dP/dt) at a frequency response of 100 Hz. The peak $dP/dt/P_{max}$ was calculated and used as an index of LV isovolumic contraction.

The right femoral artery and vein were cannulated for connection to the artificial kidney. The artificial kidney had a capillary flow dialyser (Travenol CF 12.11) which was connected with appropriate tubing to the dialysate and through a roller pump to the experimental animal. The dialyser and the tubing were filled up with heparinized 5% dextrose solution.

The animals were divided into four groups. Groups C1 and L1 underwent HD and thoracotomy only while C2 and L2 were subjected to HD and ligation-reperfusion. In groups C1 and C2 (control groups) the concentration of Ca, Mg, Na and K in the dialysate were in 2.1, 0.7, 140 and 3.6, respectively, whereas in group L1 and L2 (low calcium groups) that of Ca was 0.15 mM. The flow of the dialysate was maintained at 300 ml/min and the blood flow at 130–150 ml min.

In groups C2 and L2, after 30 min of HD, a left thoracotomy at the fourth intercostal space was performed and the left anterior descending coronary artery was isolated and ligated just distally to the first diagonal branch by complete closure of a snare for 90 min. The ligature was then released to allow reperfusion for 30 min.

Serum ionised Ca concentration was measured by a Ca^{2+}-selective electrode at 0, 30, 120 and 150 min of HD. At the end of the experiments the heart was rapidly excised, atria and connective tissue were removed and samples of left ventricular muscle were taken from non-ligated and ligated-reperfused myocardium.

Calcium concentration was determined in about 500 mg of tissue prewashed with 0.3 M sucrose and deionised water and then compressed between filter paper with 300 g load for 1.5 min [19]. After dry weight estimation, the samples were submitted to acidic digestion [20] and the concentration of calcium was measured by atomic absorption spectrophotometry.

ATP and CP were extracted from tissue samples of about 200 mg, immediately frozen and then powdered in liquid nitrogen. The powdered samples were homogenized in 1.2 ml $HClO_4$ 3 M [21]. The final neutralized supernatants of the centrifugation at $14000 \times g$ were used for ATP and CP measurements by standard enzymatic procedures [22].

Heart sarcolemma was prepared by the method of Jones et al. [23]. At the end of the procedure, the vesicles were suspended in 160 mM KCl and 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, pH 7.4). Cytochrome-*c* oxidase [24] and NADPH reductase [25] activities were measured to determine the extent of contamination with mitochondria and sarcoplasmic reticulum, respectively.

The activity of Na^+/K^+-ATPase was measured in a medium containing 3 mM $MgCl_2$, 3 mM ATP, 100 mM NaCl, 20 mM KCl, EDTA/imidazole/glycylglycine buffer (0.5 mM EDTA, 30 mM imidazole, 30 mM glycylglycine, pH 7.4) and about 10–12 mg of sarcolemmal protein at 37°C in a final volume of 0.75 ml [26]. Ouabain-sensitive Na^+/K^+-ATPase activity was taken as the activity inhibited by 1 mM ouabain. The reaction was terminated by adding ice-cold 10% (w/v) trichloroacetic acid and inorganic phosphate was determined [27] in the supernatant following centrifugation. Protein concentration was determined by the method of Lowry et al. [28]. In the experiments where the effect of increased concentrations of calcium on Na^+/K^+-ATPase activity was studied in vitro, $CaCl_2$ was added in the reaction mixture to give final concentrations in the range of 3.10^{-6} M to 10^{-3} M. Free calcium concentrations varied from $1.7 \cdot 10^{-7}$ M to $4.6 \cdot 10^{-4}$ M and were calculated [29] for pH 7.4 at a reaction temperature of 37°C by means of relevant association constants (K_{app}). These were for MgATP, 4.20, CaATP, 3.89, MgEDTA, 5.82 and CaEDTA, 7.72. The calculated free calcium values were verified by a Ca^{2+}-selective electrode.

The Student's *t*-test was used to estimate the statistical significance of the observed differences. A value of $P < 0.05$ was considered significant. The correlation coefficient (r) was calculated by regression analysis when it was necessary.

Results

Table I shows the concentrations of ionised calcium (Ca_i) in serum of group L2 at different times of hemodialysis. It is observed that low calcium HD caused a significant ($P < 0.001$) reduction of Ca_i by 50% at the end of hemodialysis. However, in groups subjected to normal calcium HD the concentration of Ca_i remained unaltered. Similar results with groups L2 and C2 were obtained in groups L1 and C1, respectively (not shown).

At the end of reperfusion, under stable serum Ca_i concentration (Table I), the results of heart rate, left ventricular systolic and left ventricular end-diastolic

TABLE I

Serum ionised calcium in dogs subjected to low-calcium hemodialysis and ligation-reperfusion

Ionised calcium (Ca_i) concentration was measured in serum by a Ca^{2+}-selective electrode at different times of hemodialysis. Each value is the mean ± S.E. of the experiments shown in parentheses.

Group of animals [a]	Time of hemodialysis (min)	Ca_i (mM)
C2 (7)	0	1.30 ± 0.03
	30	1.35 ± 0.04
	120	1.37 ± 0.06
	150	1.34 ± 0.05
L2 (6)	0	1.26 ± 0.04
	30	0.89 ± 0.02 [b]
	120	0.64 ± 0.02 [b]
	150	0.62 ± 0.02 [b]

[a] Groups C2 and L2, normal and low-calcium groups, respectively, subjected to ligation and reperfusion.

[b] Significantly different values from 0 min of hemodialysis ($P < 0.001$).

pressures (mean values ± S.E. of six or seven experiments) were 154 ± 9 beats/min, 115 ± 6 mmHg and 11.2 ± 0.9 mmHg, respectively, for group C2 and 143 ± 2 beats/min, 119 ± 4 mmHg and 10.0 ± 0.6 mmHg for group L2. The lack of significant differences of the above measurements between the two groups, allow us to compare the peak $dP/dt/P_{max}$, which did not differ significantly between group L2 (22.1 ± 0.5 s^{-1}) and group C2 (23.4 ± 1.1 s^{-1}) The similarity of all of these parameters in the normal and low calcium groups suggests that the inotropic state of the heart was not affected under the described conditions.

The decrease of serum calcium in the group of animals which did not undergo ligation (L1) caused a significant ($P < 0.01$) decrease of myocardial calcium compared to the normal calcium group C1 (Table II). As it would be expected, ligation-reperfusion caused a significant ($P < 0.01$) increase of calcium in the ligated myocardium of group C2. However, in the low calcium group L2, the concentrations of calcium in the non-ligated and ligated-reperfused myocardial areas were lower ($P < 0.02$) than the respective concentrations of the normal calcium group C2. Moreover, calcium content in the ligated myocardium of the same group did not differ from normal values (i.e. group C1).

The results of Table II also show that in the normal calcium group C2 where the accumulation of calcium was higher, ligation caused a reduction of both ATP and CP content, which was small but significant ($P < 0.05$) even in the non-ligated myocardial area. In group L2, under the conditions of decreased myocardial calcium, the reduction of ATP seems to be confined only in the ligated-reperfused area, since in the non-ligated myocardium ATP was significantly ($P < 0.01$) higher

TABLE II

Calcium, ATP and creatine phosphate in the non-ligated and ligated myocardium of dogs with normal and low-serum calcium

Tissue samples which were excised from the non-ligated and ligated-reperfused myocardial areas, were powdered in liquid nitrogen. The extraction and the determination of ATP and CP were performed as described in Materials and Methods. Data represent the mean ± S.E. of the experiments shown in parentheses.

Group of animals [a]		Content (mmol/kg dry wt.)		
		Ca	ATP	CP
C1 (6)		4.3 ± 0.3	22.8 ± 1.9	27.0 ± 2.0
L1 (6)		2.8 ± 0.2 [b]	22.2 ± 1.4	27.1 ± 1.3
C2 (7)	Non-ligated	3.9 ± 0.2	18.2 ± 0.7	20.5 ± 1.2
	Ligated-reperfused	8.9 ± 1.0 [c]	10.7 ± 1.5 [c]	14.5 ± 1.4 [c]
L2(6)	Non-ligated	2.7 ± 0.2	22.7 ± 1.2	25.9 ± 1.4
	Ligated-reperfused	5.3 ± 0.7 [c]	13.1 ± 2.2 [c]	22.5 ± 1.6

[a] Groups C1 and L1, normal and low calcium groups, respectively, not subjected to ligation; groups C2 and L2, normal and low calcium groups, respectively, which underwent ligation and reperfusion.

[b] $P < 0.01$ vs. C1.

[c] $P < 0.01$ vs. non-ligated myocardium.

than that of group C2. Regarding CP there was a significant ($P < 0.01$) 30% decrease in the ligated-reperfused area of group C2 which was abolished in the low-calcium group.

In sarcolemmal membranes isolated as described in Methods, mitochondrial and microsomal contamina-

TABLE III

Sarcolemmal ouabain-sensitive Na^+/K^+-ATPase activity in the non-ligated and ligated myocardium of dogs with normal and low myocardial calcium

Tissue samples were excised from the non-ligated and the ligated-reperfused myocardial areas. The isolation of sarcolemmal membranes and the determination of Na^+/K^+-ATPase activity were performed as described in Materials and Methods. Data represent the mean ± S.E. of the experiments shown in parentheses.

Group of animals [a]		Na^+/K^+-ATPase (μmol P_i/mg protein per h)
C1 (6)		9.6 ± 0.4
L1 (6)		12.4 ± 0.3 [b]
C2 (7)	Non-ligated	9.6 ± 0.2
	Ligated-reperfused	6.8 ± 0.3 [c]
L2 (6)	Non-ligated	11.7 ± 0.4 [d]
	Ligated-reperfused	8.4 ± 0.7 [c]

[a] Groups C1 and L1, normal and low calcium groups, respectively, not subjected to ligation; groups C2 and L2, normal and low calcium groups, respectively, which underwent ligation and reperfusion.

[b] $P < 0.001$ vs. C1.

[c] $P < 0.01$ vs. non-ligated myocardium of the same group.

[d] $P < 0.002$ vs. non-ligated of group C2.

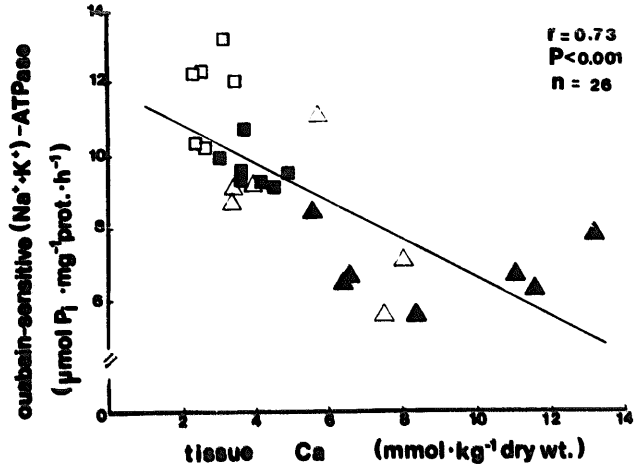

Fig. 1. Correlation between sarcolemmal ouabain-sensitive Na^+/K^+-ATPase activity and myocardial calcium. The following symbols are used: ■ and ▲ for the non-ligated and ligated-reperfused myocardium of normal calcium group, respectively; □ and △ for the non-ligated and ligated-reperfused myocardium of low calcium group, respectively.

tions were negligible because cytochrome-*c* oxidase and NADPH–cytochrome-*c* reductase were 0.2-times those in crude homogenates. Furthermore, Na^+/K^+-ATPase activity in the sarcolemmal preparations was enriched in the homogenates by 26 times. Approximately 25% of the sarcolemmal Na^+/K^+-ATPase was inhibited by 1 mM ouabain. Protein yield in the sarcolemmal fractions was about 0.2 mg g^{-1} wet tissue.

Low myocardial calcium caused a significant ($P < 0.001$) increase in the activity of ouabain-sensitive Na^+/K^+-ATPase in group L1 compared to C1 (Table III). In groups subjected to ligation and reperfusion, Na^+/K^+-ATPase activity was protected under low calcium. This is shown by its higher levels in the non-ligated myocardium and by its normal value in the ligated-reperfused area (Table III). Moreover, the regression analysis between Na^+/K^+-ATPase activity and myocardial calcium in the C2 and L2 groups (Table II) which reveals a highly significant ($P < 0.01$) negative correlation (Fig. 1), provides additional evidence supporting the inverse relation between these two parameters.

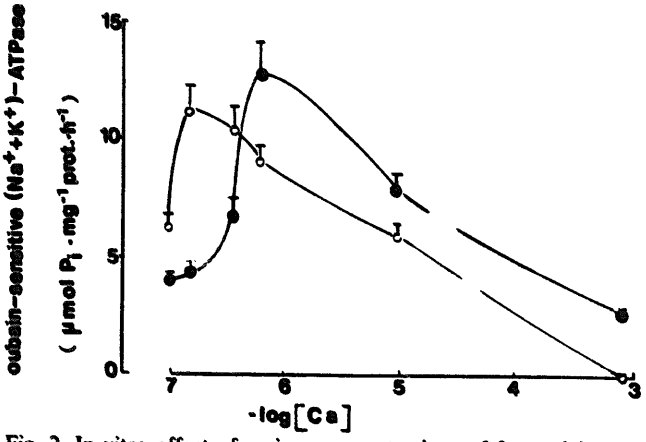

Fig. 2. In vitro effect of various concentrations of free calcium on sarcolemmal ouabain-sensitive Na^+/K^+-ATPase activity. Each value represents the mean ± S.E. of three experiments. The symbols ○ and ● are used for normal and low calcium groups, respectively, which were not subjected to ligation.

In accordance to the in vivo effect of calcium on Na^+/K^+-ATPase activity, it was shown (Fig. 2) that low concentrations of added calcium activate the enzyme in vitro. It is evident that the peak of Na^+/K^+-ATPase activation is observed at higher concentration of calcium ($6.3 \cdot 10^{-7}$ M) in the low calcium group L1 than in the normal calcium group C1 ($1.6 \cdot 10^{-7}$ M). As expected, high concentrations inhibit Na^+/K^+-ATPase and 50% inhibition is achieved at greater concentration of calcium in group L1 than in group C1.

Discussion

It is generally accepted that reperfusion after prolonged ischemia is associated with a large increase in calcium content in severely damaged myocardium [9–11]. Increased calcium concentration correlates with depressed generating capacity of ATP during reperfusion [11,30], since the activity of certain enzymes that regulate aerobic energy metabolism is strictly dependent on a narrow range of calcium concentrations [31]. The results of this paper show that the reduction of calcium in the non-ligated and ligated-reperfused myocardium of group L2, exerts protective effect on the high energy phosphates. This is evident from the values of CP in the ligated myocardial area which fluctuate within normal range. Moreover, the above-mentioned conclusion can be supported by the increased concentrations of ATP in the non-ligated and ligated-reperfused myocardium compared to those of the normal calcium group. Since the ATP content of the ligated area in the former group is still significantly ($P < 0.01$) lower than that of the non-ligated area while CP is completely restored in both of these areas, we may infer that during low calcium reperfusion, regenerated ATP is further utilized for the formation of CP.

The extent of ATP restoration and the complete recovery of CP compare favourably with the results concerning the use of calcium blockers and low calcium perfusate in ischemic heart preparations [13–15]. The experimental procedure followed in this study shows that the application of modified HD may be a beneficial intervention for the myocardial high energy phosphates in whole experimental animals, as in this case the depletion of the above compounds is further accelerated by electrical activation and accompanying mechanical response [2], compared with the case of ischemic heart preparations.

It is known that prolonged ischemia causes a considerable decrease in sarcolemmal Na^+/K^+-ATPase ac-

tivity [5,6]. Dhalla et al. [7] found that this decrease is further exacerbated during reperfusion probably as a result of the excessive intracellular calcium entry. However, there is no evidence concerning the modulation of Na^+/K^+-ATPase activity by calcium in the ischemic-reperfused myocardium. The results of this study showed that the decrease of myocardial calcium in group L2 abolishes the inhibition of Na^+/K^+-ATPase activity in the ligated-reperfused area. Furthermore, we observed a negative correlation ($P < 0.001$) between myocardial calcium concentration and sarcolemmal Na^+/K^+-ATPase activity. This probably suggests that, in vivo, calcium binds to Na^+/K^+-ATPase or to regulatory proteins according to its myocardial concentration, and affects the enzyme activity. The above suggestion can be justified by preliminary studies which showed that intracellular proteins such as calmodulin and calnaktin, interact with calcium and potentiate its activatory or inhibitory effect at low or high concentrations, respectively [32]. Further support for the modulating effect of calcium on Na^+/K^+-ATPase activity is provided by the in vitro experiments of this study (Fig. 2). It was shown that in the sarcolemmal membranes of calcium-depleted hearts (group L1), the concentration of calcium required for the maximum activation of Na^+/K^+-ATPase is 4-fold higher than that in the membranes of the normocalcemic hearts. These results may be indicative of how low calcium bound to the membranes of group L1, differentiates the activity of Na^+/K^+-ATPase from that of group C1, at various concentrations of free calcium. Besides, as the 50% inhibition of Na^+/K^+-ATPase in the low calcium group L1 occurs in vitro at higher concentrations of calcium ($3 \cdot 10^{-5}$ M) than in the normal calcium group C1 ($1 \cdot 10^{-5}$ M), we may infer that under conditions of ischemia-reperfusion, greater concentrations of intracellular calcium inhibit the enzyme in the former group. We should note that Godfraid et al. [33] also found depressed Na^+/K^+-ATPase activity at concentrations higher than 10^{-5} M. Huang and Askari [34] suggested that the inhibition of Na^+/K^+-ATPase under these concentrations, may be a result of the competition between magnesium and calcium for the phosphorylated form of the enzyme, and may occur under pathological conditions such as ischemia.

In conclusion, this study compares the effect of low to normal myocardial calcium on the decrease of high energy phosphates, which was caused by coronary ligation and reperfusion in whole animals. Furthermore, it provides evidence for the beneficial modulation of ouabain-sensitive Na^+/K^+-ATPase activity by low calcium in the sarcolemmal membranes isolated from normal and infarcted heart areas. The latter is of great importance since Na^+/K^+-ATPase contributes to the preservation of the intracellular electrolyte balance, which is severely disturbed under the conditions of ischemia and reperfusion.

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