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THE NATURE OF THE CARDIAC RELAXING FACTOR*

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SUMMARY

In the presence of cardiac reticulum parallel measurements of the pCa of the medium and the inhibition of myofibrillar activity, *i.e.* syneresis and ATPase activity, were made under a variety of conditions. It was found that the dependence of myofibrillar activity on pCa was not altered by the presence of cardiac reticulum, *i.e.* cardiac reticulum apparently causes relaxation only by the removal of Ca^{2+} and not by any direct action on the myofibrils. The capacity of cardiac reticulum for Ca^{2+} appears to be sufficient to account for relaxation *in vivo*.

INTRODUCTION

It has been suggested that cardiac reticulum causes relaxation through the action of a soluble relaxing substance[1–5]. However, it has been demonstrated that cardiac myofibrils and actomyosin require Ca^{2+} for syneresis[6–9] and that cardiac reticulum accumulates Ca^{2+} (refs. 8–14). Furthermore, FANBURG *et al.*[8] showed that cardiac reticulum prevents syneresis only when it lowers the level of Ca^{2+} in the medium to 0.3 μM and not if Ca^{2+} remains at 9 μM.

If cardiac reticulum were to cause relaxation by means other than the removal of Ca^{2+} from the myofibrils, one would expect that cardiac reticulum alters the correlation between the pCa of the medium and syneresis and ATPase activity of the myofibrils. Therefore, we studied under a variety of conditions the dependence of myofibrillar syneresis and ATPase activity in the presence and in the absence of cardiac reticulum. Some of these data have been presented previously[10,15].

METHODS

Myofibrils were prepared from rabbit skeletal muscle and stored in glycerol as described previously[16]. One day preceding the experiment they were treated with 1 mM $MgCl_2$ and 1 mM EGTA at pH 7 to remove as much of the bound Ca^{2+} as possible[6]. Mg^{2+} and EGTA were removed by subsequent washes with 0.1 M KCl. About

Abbreviations: PEP, phosphoenolpyruvate; EGTA, ethyleneglycol bis-(β-aminoethyl-ether)-*N*,*N*-tetraacetic acid.

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1 μmole Ca per g myofibrillar protein remained bound. This treatment was introduced so as to avoid altering significantly the specific activity of ^{45}Ca in the assays when the myofibrils were added at the end of the preincubation period.

Cardiac reticulum was prepared from dog heart and in one instance from the hearts of dystrophic chickens. The hearts were cut fine with scissors and then treated with a blendor for 40–60 sec in 3–4 vol. of a solution containing 0.1 M KCl and 1 mM histidine. Afterwards, the preparation was continued as described previously for skeletal reticulum[7]. The fraction sedimenting between 8000 and 36000 $\times g$ was collected. Generally, the preparations were used on the same day, but occasionally, on the following day.

The protein concentration was determined according to LOWRY *et al.*[17], read against a curve standardized by Kjeldahl.

Ca^{2+} removal from the medium or Ca^{2+} uptake by the reticulum was determined from the disappearance of ^{45}Ca from the medium as described previously[18]. Because myofibrils were also present in these assays, prefilters in addition to the 0.45 HA filters were used for the separation of the medium. Under the conditions of the assay, the prefilters did not retain any ^{45}Ca. In contrast to skeletal preparations, the content of endogenous Ca^{2+} in the cardiac reticulum was negligible (3–6 mμmoles per mg protein).

The assay conditions for the measurement of syneresis and myofibrillar ATPase activity were identical to those for the measurement of Ca^{2+} removal from the medium except that ^{45}Ca was omitted. Syneresis was determined as described previously[7,19]. The centrifugation time was 1 min.

The ATP hydrolysed by myofibrils in the presence of reticulum was obtained by subtracting from the total value the ATP hydrolysed by reticulum alone in a parallel assay with the myofibrils replaced by 0.1 M KCl. In the presence of PEP P_i was measured according to TAUSSKY AND SCHORR[20]. In the presence of creatine phosphate creatine in the filtered supernatant (0.45 HA Millipore) of the incubation assay was determined by a procedure based on the work by TANZER AND GILVARG[21] measuring the oxidation of NADH at pH 9.1 (20 mM Tris buffer) resulting from the following reaction sequence:

$$\text{creatine} + \text{ATP} \xrightarrow[\text{kinase}]{\text{creatine}} \text{creatine phosphate} + \text{ADP} + \text{PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{ATP} +$$

$$\text{pyruvate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + \text{NAD}^+$$

The concentration of the components in the final measuring assay was the following: 1.3 mM ATP, 3.4 mM PEP, 0.13 mM NADH, 0.08 mg creatine kinase/ml, 0.02 mg pyruvate kinase/ml, 2.1 mM $MgCl_2$, 40 mM KCl.

The pCa values were calculated from the ratios Ca-EGTA/EGTA only, neglecting the Ca^{2+} binding by other components. Because the assays in the presence and in the absence of reticulum were identical and because we were only interested in a comparison this simplified procedure is justifiable although the pCa values thus obtained are too low.

RESULTS

In the experiment described in Fig. 1, the cardiac reticulum was incubated with Ca^{2+} and ATP in the presence and in the absence of oxalate for varying periods of

time before the myofibrils were added to measure syneresis. In the absence of oxalate, Ca^{2+} uptake was delayed for about 10 min and then proceeded slowly whereas in the presence of oxalate Ca^{2+} was removed from the medium much more rapidly (left side Fig. 1). A parallelism between the removal of Ca^{2+} from the medium and the inhibition of syneresis can be seen: syneresis is inhibited after a short preincubation in the presence of oxalate and the inhibition is delayed in its absence (Fig. 1, right side).

In the absence of oxalate, Ca^{2+} removal was measured after preincubation with reticulum alone and under conditions identical to the syneresis assay *i.e.* with myofibrils present during the last 45 sec of the incubation period. In the latter case, more Ca^{2+}

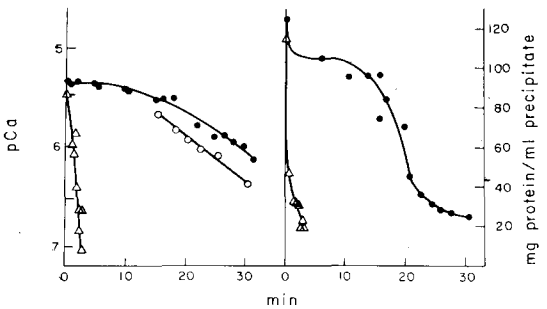

Fig. 1. The effect of various periods of incubation of cardiac reticulum with Ca^{2+} and ATP on the pCa of the medium and on myofibrillar syneresis. Left pCa, right syneresis. Experimental conditions identical for the measurement of both syneresis and pCa, except that during one set of pCa measurements (● left side) myofibrils were not present during the last 45 sec of the incubation period. Incubation mixture and conditions: 0.29 mg reticular protein per ml, 1.25 mg myofibrillar protein per ml, 2 mM MgATP, 10 mM creatine phosphate, 0.1 mg creatinephosphokinase per ml, 0.02 mM EGTA, 0.036 mM $^{45}CaCl_2$; I = 0.08; 20 mM imidazole; pH 6.6; 10 ml. Left and right: △, 1 mM oxalate; ●, ○, no oxalate.

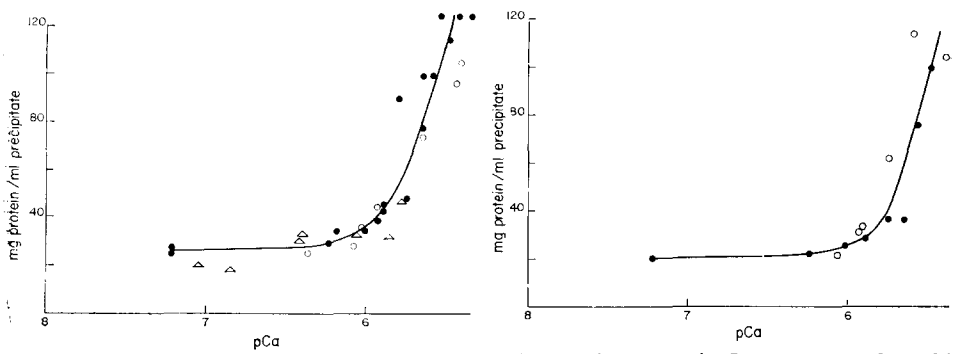

Fig. 2. Data of Fig. 1 plotted to represent syneresis as a function of pCa as compared to this function in the absence of reticulum. ○, △, data from Fig. 1; △, 1 mM oxalate; ○, no oxalate, pCa values measured in the presence of myofibrils were used; ●, no reticulum present, pCa adjusted by varying the Ca-EGTA/EGTA ratios; total EGTA 0.02 mM, incubation mixture identical to that used in the presence of reticulum.

Fig. 3. Syneresis as a function of pCa in the absence and in the presence of reticulum preincubated for 5 min with varying amounts of Ca^{2+} (110–217 mμmoles per mg reticular protein, 0.23 mg reticular protein per ml). Myofibrils incubated for 30 sec with reticulum before centrifugation (for 60 sec). pCa as measured at the end of 30-sec incubation of myofibrils with reticulum or – in the absence of reticulum – as adjusted by the Ca-EGTA/EGTA ratio. Reticulum: ○, present; ●, absent. Incubation mixture for both conditions: 1.6 mg myofibrillar protein per ml, 1 mM oxalate, 1 mM MgATP, 10 mM creatine phosphate, 0.1 mg creatinephosphokinase per ml, 20 μM total EGTA, 20 mM imidazole, pH 6.6; 10 ml.

was removed from the medium. This was probably due to the fact that as a consequence of myofibrillar ATP hydrolysis the phosphate level in the medium was higher. Presumably in the absence of oxalate, the uptake of Ca^{2+} by the reticulum became significant only when, as a result of ATP hydrolysis by the reticulum, the phosphate level in the medium had risen to values where calcium phosphate precipitation in the interior of the vesicles occurs.

When the correlation between pCa and syneresis in this experiment was compared with that in the absence of reticulum (Fig. 2) the correlation was found to have remained unchanged by the presence of the reticulum. When we measured the inhibition of myofibrillar syneresis in the presence of reticulum preincubated for a constant period of time but with varying concentrations of Ca^{2+}, myofibrillar activity again depended only on the extent to which reticulum had lowered the level of Ca^{2+} in the medium. This is indicated by the fact that the correlation between pCa and syneresis had remained the same as in the absence of reticulum (Fig. 3).

In the experiment shown in Fig. 4, we added Ca^{2+} buffer to the medium in a sufficiently high concentration so that the removal of the small amount of Ca^{2+} which the reticulum was capable of accumulating, could not significantly raise the pCa. Under these conditions the preincubation of the assay system with reticulum for 5 min had no significant effect on the ATPase activity of the myofibrils.

However, when we repeated this experiment with another preparation without first determining its capacity for Ca^{2+} uptake and preincubated with reticulum for 10

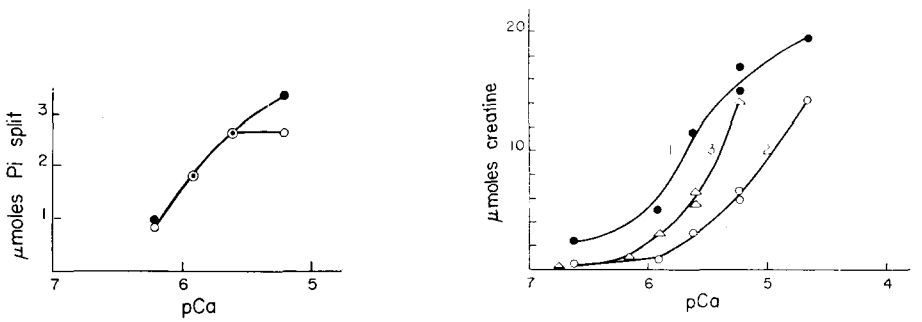

Fig. 4. Myofibrillar ATPase activity as a function of pCa in the absence (●) and in the presence (○) of reticulum after 5 min incubation. Because Ca^{2+} buffer of varying pCa had been added in very high concentration (3 mM) and because the reticulum could take up only 80 mμmoles of Ca^{2+} in 5 min it was assumed that the pCa of the medium was not altered by incubation with the reticulum. Incubation mixture and conditions: 0.61 mg reticular protein per ml when present, 2.45 mg myofibrillar protein per ml, 1 mM oxalate, 1 mM pyrophosphate, 10 mM phosphoenolpyruvate, 0.01 mg phosphoenolpyruvate kinase per ml, 1.6 mM $MgCl_2$, 0.5 mM ATP; $I = 0.11$; 15 mM imidazole, pH 6.6; 1.0–2.5 mM total Ca; 24.5°. The inorganic phosphate liberated by the myofibrils in 30 sec was measured after the termination of the assay by trichloroacetic acid.

Fig. 5. Myofibrillar ATPase activity as a function of pCa in a strongly Ca^{2+}-buffered medium before (Curve 1) and after preincubation with reticulum of a high capacity for Ca^{2+} uptake (Curves 2 and 3). Preincubation with 0.64 mg reticular protein per ml for 10 min, followed by 2 min incubation with myofibrils and termination of the assay by centrifugation for 90 sec. Curve 2: pCa values as calculated from the Ca-EGTA/EGTA ratio before incubation with reticulum; Curve 3: pCa calculated by taking into account the Ca^{2+} uptake by the reticulum measured on the next day (Fig. 6). Incubation mixture and conditions: 1.58 mg myofibrillar protein per ml, 2 mM oxalate, 0.96 mM ATP, 2.02 mM $MgCl_2$, 0.96 mM pyrophosphate, 9.6 mM creatine phosphate, 0.1 mg creatine phosphokinase per ml, 2.9 mM total EGTA, 0.5–2.9 mM total Ca; $I = 0.125$; 14 mM imidazole, pH 6.5; 10.4 ml; 24.5°.

min, we found the myofibrillar ATPase activity greatly reduced (Fig. 5, Curve 2). This finding was explained by the relatively high capacity for Ca^{2+} uptake exhibited by this preparation (Fig. 6), even one day later. When the pCa values for the end of the incubation period were calculated on the basis of the uptake curves of Fig. 6, the correlation between pCa and ATPase activity in the presence of reticulum differed less markedly from that in its absence. The finding that the inhibition of myofibrillar ATPase activity nevertheless occurred at lower pCa values than in the absence of reticulum may have resulted from the fact that the pCa values were based on Ca^{2+} uptake measurements performed 1 day after the experiment. These rates of uptake may have been too low since it has usually been found that the rate of Ca^{2+} uptake by cardiac reticulum deteriorates rather rapidly from day to day.

Consistent with these data indicating that there is no evidence for a soluble relaxing substance, myofibrillar ATPase activity was found not to be altered by the addition of supernatants of media which had been incubated with reticulum and ATP (Table I). We compared the effect of incubation in the presence and in the absence of pyrophosphate because in some preparations pyrophosphate had been found to increase reticulum activity. In those cases, Ca^{2+} uptake was increased although oxalate was also present. With other preparations, however, it had no effect in the presence of oxalate (Fig. 7). When, instead of the supernatant, the whole incubation

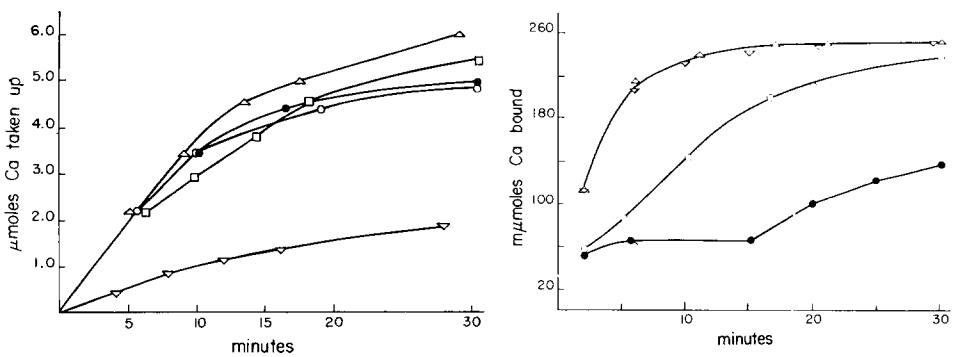

Fig. 6. Ca^{2+} uptake by the reticulum used in the experiment of Fig. 5. Same conditions except that the total EGTA varied between 0.62 and 3.0 mM and the total Ca between 0.5 and 1.0 mM.

Values for Ca^{2+} (μM) after increasing periods of Ca^{2+} uptake

Time (min)	○	△	□	●	▽
0	6.0	2.4	1.2	35.0	0.24
0–5	1.3	2.2			0.22
5–10	0.65	0.93	0.78 0.66	0.8	0.20
10–15		0.68	0.54	0.41	0.18
15–20	0.34	0.6	0.44		0.17
25–30	0.21	0.43	0.35	0.23	0.14

Fig. 7. Ca^{2+} uptake by cardiac reticulum. 0.4 mg reticular protein per ml, 20 mM imidazole, pH 6.6, $I = 0.092$, 1.0 mM ATP, 2.1 mM $MgCl_2$, 0.1 mM Ca-EGTA, 10 mM creatine phosphate, 0.1 mg creatinephosphokinase per ml. ●, no other additions; ○, 1.0 mM pyrophosphate; △, 2.0 mM oxalate; ▽, 2.0 mM oxalate + 1.0 mM pyrophosphate; △, ▽, Ca^{2+} 0.034 μM after 30 min.

mixture including the reticulum was added to the myofibrils, myofibrillar ATPase activity was inhibited.

In agreement with other investigators[8,11], we found that cardiac reticulum takes up much less Ca^{2+} than preparations from skeletal muscle and that its ability for Ca^{2+} accumulation deteriorates rapidly. Our best preparation from the hearts of dystrophic

TABLE I

EFFECT ON MYOFIBRILLAR ATPASE ACTIVITY OF THE SUPERNATANTS FROM SOLUTIONS INCUBATED WITH VARYING AMOUNTS OF RETICULUM AND ATP

Various concentrations of reticular protein (0.49, 0.97 and 1.94 mg/ml) were incubated for 14 min in a medium containing 20 mM imidazole, pH 6.5, I 0.088, 1.0 mM MgATP, 1.0 mM oxalate, 1.0 mM pyrophosphate when present, 5 mM PEP and 0.01 mg pyruvate kinase per ml. After centrifugation for 20 min at 100000 × g 4 ml supernatant were added to a 10-ml assay containing 20 mM imidazole, pH 6.5, 2.0 mM MgATP, 5 mM PEP, 0.01 mg pyruvate kinase per ml, 1 mM EGTA + Ca-EGTA of varying ratio, I 0.11 for 0.19 and 0.38 corresponding reticulum concentration and I 0.14 for 0.78; 1.3 mg myofibrillar protein per ml.

Control	*ATPase activity, μmoles P_i split Supernatant, corresponding to a final concentration of reticulum in mg per ml ATPase assay system*			*Control*	*Ca^{2+} (μM)*	*Pyrophosphate present in incubation medium*
	0.19	*0.38*	*0.78*			
4.23	4.19	4.12			1.8	—
	4.32	3.89			1.8	+
2.67	2.51	2.39			0.8	—
	2.69	2.27	1.61	1.73	0.8	+
1.85	1.77	1.80			0.51	—
	1.73	1.73			0.51	+
			2.53	2.57	No Ca^{2+} added	+

chickens in the absence of oxalate accumulated about 53 mμmoles Ca per mg reticular protein in 2 min, *i.e.* before calcium phosphate precipitation in the interior of the vesicles occurred (Fig. 7). The second rise in Ca^{2+} uptake is probably caused by phosphate accumulation in the medium and calcium phosphate precipitation in the interior of the vesicles. The uptake at 53 mμmoles per mg protein is only about a quarter of the amount taken up by the best of our skeletal preparations[18]. However, such a capacity for Ca^{2+} could be adequate to account for relaxation *in vivo* according to the following consideration. In this instance 2.4 mg reticular protein were isolated per g wet weight muscle. If we assume a 50 % yield, which is probably too high, 1 g wet weight of muscle would have contained 4.8 mg, or a total capacity of the reticulum for Ca^{2+} of 0.25 μmole. Since earlier studies on skeletal myofibrils indicated that about 1 μmole Ca^{2+} must be removed from 1 g myofibrillar protein to obtain relaxation and since 1 g muscle contains about 100 mg myofibrillar protein, this Ca^{2+} capacity would be twice that necessary for relaxation. However, under the conditions of this experiment, the pCa of the medium was not raised to 7, the level necessary for the dissociation of myofibrillar Ca^{2+}. Therefore, this consideration applies only if, for cardiac reticulum, the capacity for Ca^{2+} remains nearly maximal up to pCa 7 as it does for skeletal reticulum[10,18], or at least does not fall much below half. This has not been investigated for cardiac reticulum.

DISCUSSION

The experiments presented here indicate that cardiac reticulum inhibits myofibrillar activity by lowering the concentration of ionized Ca in the medium and they do not give evidence for any other kind of relaxing effect, *e.g.* a soluble relaxing substance.

We used in this study skeletal rather than cardiac myofibrils for the following reasons. First, only part of the ATPase activity of cardiac myofibril preparations can be attributed to actomyosin; most of it is probably a property of other contaminating subcellular fractions[8]. Second, most of our cardiac myofibrils had contraction bands after preparation and therefore the volume change on syneresis was much smaller than the volume change exhibited by skeletal myofibrils. We found the volume change (about 2-fold) too small to study the intermediate stages of inhibition of myofibrillar syneresis with confidence.

We do not know why the myofibrils used in this study required a higher concentration of ionized Ca^{2+} for activation than the myofibrils used in previous studies[6,22]. From control experiments we can exclude as a reason the presence of oxalate, pyrophosphate or a high concentration of creatine phosphate.

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