BBA 46068

MITOCHONDRIAL CALCIUM UPTAKE IN THE PERFUSED CONTRACTING RAT HEART AND THE INFLUENCE OF EPINEPHRINE ON CALCIUM EXCHANGE

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(Received July 31st, 1970)

SUMMARY

Perfused rat hearts were exposed to solutions containing $^{45}Ca^{2+}$ with and without epinephrine. They were subjected to differential centrifugation and the distribution of Ca and ^{45}Ca in mitochondria and microsomes was determined. It was found that the mitochondria contain most of the calcium of the intact heart and that the exchange of mitochondrial calcium with extracellular calcium was extremely rapid. This process was accelerated in hearts stimulated by epinephrine.

INTRODUCTION

Ca^{2+} has been shown to be the agent which initiates muscle contraction[1] and relaxation can occur only when the calcium concentration within the myoplasm falls below a certain level[2–5]. In skeletal muscle excitation-contraction has been shown to be mainly a function of the sarcoplasmic reticulum and this cellular component is also important in the binding of calcium which is necessary to produce relaxation.

Cardiac muscle differs from skeletal muscle in several important respects. The cells are smaller than those of skeletal muscle and the duration of a single contraction is far longer. It has been calculated that in skeletal muscle cell the calcium entering the cell cannot diffuse to the myofibrils quickly enough to account for the initation of the muscle twitch. Calcium entering the skeletal muscle cell is believed not to elicit contraction directly while in the myocardium diffusion of calcium from the extracellular space to the contractile proteins is thought to be the process that triggers muscle shortening[4,5].

There is considerable experimental evidence that factors other than transport of calcium across the cell membrane are involved in the regulation of the myocardial calcium fluxes and the contractile cycle of the heart. Heart microsomes, like microsomes from skeletal muscle, are capable of sequestering calcium[6–8]. The sarcoplasmic reticulum, though not as extensive as in skeletal muscle, may therefore play a role in the relaxation of the myocardium. Another cellular organelle that should be con-

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sidered as a possible part of the relaxing system is the mitochondrion. Heart mitochondria are situated close to the myofibrils. They have been shown to accumulate calcium rapidly when incubated *in vitro*[7,9–12] and can lower the concentration of calcium in the suspension medium to a value below that necessary to produce relaxation *in vivo*[13]. PATRIARCA AND CARAFOLI[14] have shown recently that, in the intact rat, intraperitoneal injection of radioactive calcium leads to more extensive labeling of the mitochondria than any other cellular component of the heart. We have studied the effect of oligomycin on the isolated, perfused rat heart[15]. This compound inhibits the ATP-supported uptake of calcium by mitochondria but has little or no effect on the binding of calcium by microsomes[7]. Inclusion of oligomycin in the perfusion medium inhibited the ability of the myocardium to relax when the heart was stimulated electrically or following administration of epinephrine or theophylline. Under these conditions contracture was produced.

The experiments to be presented here are an extension of our earlier studies and were designed to determine which cellular components are most actively involved in calcium exchange in the contracting heart. The results support the view that mitochondria are intimately involved in the contraction cycle of the myocardium.

The mechanism of the inotropic action of epinephrine is at present not understood but may involve an effect of the catecholamine itself or of cyclic 3′,5′-AMP on calcium movements in the cell[16]. Experiments were, therefore, carried out also with hearts stimulated by epinephrine.

METHODS

Heart perfusion

Male Wistar rats weighing between 250 and 350 g were killed by decapitation. The hearts were quickly removed and washed in Krebs–bicarbonate medium of the following composition: 119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 5 mM glucose. The medium was equilibrated with O_2–CO_2 (95:5, by vol.). Each heart was weighed and a glass cannula was placed into the aorta. The hearts were mounted on a Langendorff apparatus and perfused with the Krebs–bicarbonate solution at 38° as previously described[15]. Radiocalcium and epinephrine were infused at a rate of 1 ml/min through a catheter which reached into the tip of the cannula. $^{45}CaCl_2$ was diluted with 0.9 % NaCl to yield a solution giving approx. 275000 counts/min per ml. This was used as the infusion medium for control hearts. When required, epinephrine bitartrate, at a final concentration of 10 μg/ml, was included in this solution. All hearts were perfused for a 15-min equilibration period and then infused with one of the above solutions for 10 sec. At the end of this period they were quickly dropped into ice-cold 0.4 M sucrose containing oligomycin (2 μg/ml) and rotenone (1 μg/ml). These amounts of olimogycin and rotenone lead to concentrations in the homogenate that are several times those which we have found to completely inhibit Ca^{2+} uptake by isolated heart mitochondria incubated with ATP and glutamate (R. S. HORN, unpublished observations).

Separation of subcellular fractions

The procedure of PATRIARCA AND CARAFOLI[14] was followed with slight modifications. Each heart was minced with scissors and transferred to a centrifuge tube. The

mince was washed four times with 10 ml of 0.4 M deionized sucrose containing oligomycin and rotenone. 10 % homogenates were prepared by hand with this solution in a glass Potter–Elvehjem homogenizer with a loose-fitting pestle. An aliquot was removed and rehomogenized in a Dounce homogenizer with a tight-fitting pestle. The remaining homogenate was then subjected to differential centrifugation at 800 × *g* for 7 min, 15000 × *g* for 10 min, 30000 × *g* for 20 min and finally 105000 × *g* for 60 min. The pellets were resuspended in 0.25 M deionized sucrose also containing oligomycin (2 μg/ml) and rotenone (1 μg/ml) using the Dounce homogenizer. The fractions were designated whole homogenate (WH), residue (R), mitochondria (M), light mitochondria (LM), microsomes (Mic) and soluble fraction (S).

Calcium and radiocalcium analysis

Aliquots of the heart fractions, sucrose solutions and infusion media were dried and ashed at 550° overnight in fused silica crucibles. The ash was dissolved in 0.1 M HCl–0.01 M $SrCl_2$. Aliquots of these solutions were analyzed for calcium with a Perkin Elmer Model 303 atomic absorption spectrophotometer. Additional samples were dried in planchets and counted in a Picker low background counter. A minimum of 250 counts above background was obtained and each sample was counted at least four times.

Protein

Protein was determined by the biuret method according to CLELAND AND SLATER[17].

Cytochrome oxidase

Cytochrome oxidase activity was measured spectrophotometrically as described by WHARTON AND TZAGOLOFF[18].

Ali-esterase

The rate of hydrolysis of *p*-nitrophenyl acetate by the various heart fractions was determined manometrically with a Warburg apparatus as described by HULSMANS[19]. The rate of production of CO_2 was followed for 30 min and was linear for at least 10 min with all samples.

Statistical significance of the data was calculated by the use of Student's *t* test. Oligomycin was obtained from Sigma Chemical Company and consisted of approx. 15 % oligomycin A and 85 % oligomycin B.

RESULTS

Effect of epinephrine on contractile force

The infusion of epinephrine for 10 sec led to a marked increase in the force of contraction. At the end of this period the heart had about reached the maximum increase in contractility seen with epinephrine[20].

Separation of subcellular components

The differential centrifugation technique used in these studies does not produce pure fractions but rather gives preparations greatly enriched in a particular cell component. To localize mitochondria in the various fractions we have utilized cytochrome

oxidase activity and, as a marker for microsomes, Ali-esterase. The spread of these activities within our fractions is presented in Table I. If one accepts the assumptions of HULSMANS[19], it is possible to calculate the concentration of mitochondria and microsomes within each fraction. These assumptions are: (1) cytochrome oxidase is present only in the mitochondria; (2) the microsomal and mitochondrial fractions contain only microsomes and mitochondria; (3) the soluble fraction does not contain microsomes; (4) soluble protein is present only in the S fraction; (5) esterase is present in both the microsomal and soluble fractions. From the formula used by HULSMANS[19] it was calculated that the fractions we obtained has the distribution shown in Table II.

It can be seen that about half of the total mitochondria and microsomes were associated with the R fraction. Most of the remaining mitochondria were found in the M fraction and comprised 80 % of that material. The microsomes were found to be spread throughout the various fractions to a greater degree than the mitochondria. They were, however, isolated in the microsomal fraction (Mic) in a relatively pure state (78 %). The fraction designated LM was found to have a small protein content and was the most heterogeneous of the fractions. It was largely microsomal in nature but contained some mitochondria and a small amount of material which appeared to be neither mitochondria nor microsomes. The soluble fraction was free of cytochrome oxidase activity, indicating the absence of mitochondria and mitochondrial fragments.

TABLE I

SUBCELLULAR DISTRIBUTION OF CYTOCHROME OXIDASE AND ALI-ESTERASE IN RAT HEART

Fraction	*Cytochrome oxidase* (ΔA/*min per mg protein*)	*Ali-esterase* ($\mu l\ CO_2$/*mg protein per h*)
WH	2.12 ± 0.07*	48.2 ± 7.1*
R	1.32 ± 0.05	34.3 ± 4.4
M	5.80 ± 0.14	29.7 ± 3.7
LM	1.31 ± 0.23	87.6 ± 14.4
Mic	1.58 ± 0.28	112.6 ± 18.3
S	0	63.8 ± 15.8

* Mean ± S.E.

TABLE II

CALCULATED DISTRIBUTION OF MITOCHONDRIA AND MICROSOMES IN RAT HEART

Fraction	*% of total homogenate protein*	*% of total mitochondria*	*% of total microsomes*	*% of fraction which is*	
				Mitochondria	*Microsomes*
R	55	44	54	18	24
M	13	46	11	80	20
LM	5	4	14	18	61
Mic	6	6	20	22	78
S	20	0	0	0	0

Intracellular calcium distribution

Data concerning the distribution of calcium within the myocardium are presented in Table III. Of the fractions studied only the mitochondria were found to have a higher concentration of calcium than the whole homogenate. The soluble portion of the heart homogenate was significantly lower in calcium concentration than the WH fraction. Epinephrine was found to have no effect on either whole heart calcium concentration or on the distribution of calcium among the myocardial homogenate fractions. Correction of the data for contamination of fractions by mitochondria and microsomes (adjusted values) did not change the pattern of calcium distribution.

TABLE III

SUBCELLULAR CALCIUM DISTRIBUTION IN RAT HEART

The data for protein and calcium content was corrected for the distribution of mitochondria and microsomes throughout the various fractions according to the distribution shown in Table II. Data for total calcium and adjusted Ca/mg protein were calculated from the mean of both saline and epinephrine infused hearts since there was no statistically significant difference between these.

Fraction	*Calcium (μmoles $\times 10^3$/mg protein)*		*Total calcium (μmoles $\times 10^3$/g wet wt. heart)*
	Saline infusion	*Epinephrine infusion*	
WH	6.43 ± 0.96 (9)*	6.10 ± 1.03 (7)	1067
R	6.86 ± 0.42 (8)	5.72 ± 0.39 (7)	457
M	12.90 ± 1.46 (9)**	13.64 ± 0.96 (8)**	373
LM	6.45 ± 0.78 (9)	5.78 ± 0.68 (8)	61
Mic	6.98 ± 1.15 (8)	7.26 ± 1.36 (8)	71
S	3.36 ± 0.99 (8)***	2.31 ± 0.76 (7)**	75
	Data adjusted for distribution of mitochondria and microsomes		
Residue	4.08		172
Mitochondria	16.40		652
Light mitochondria	5.40		11
Microsomes	5.95		186
Soluble	2.85		75

* Mean ± S.E. Number in parentheses = number of samples.
** $P < 0.01$ compared to WH.
*** $P < 0.02$ compared to WH.

The total calcium content of each fraction is also presented in Table III. When correction for the spread of mitochondria and microsomes was applied to this data marked adjustments resulted. The dominance of the mitochondrial calcium pool is very evident. Most of the cardiac calcium content was located in this fraction. It was found that the microsomes also contain a sizable calcium pool, approximately equal to the calcium bound to the R fraction and amounting to about 25 % of the mitochondrial calcium pool.

GROSSMAN AND FURCHGOTT[21] have determined that the ^{45}Ca space of contracting auricles bathed in a medium containing a calcium concentration similar to that which we have used is about 40 ml/100 g wet wt.

Using the value of 1.07 μmoles Ca^{2+}/g wet wt. and an extracellular Ca^{2+} concentration of 2.6 μmoles/ml we find the same value for the calcium space of the intact heart. The total calcium was close to that of 0.86 μmoles/g reported by FEHMERS[13] for the perfused rat heart.

Studies with radioactive calcium

Infusion with isotonic NaCl-containing $^{45}CaCl_2$ for 10 sec led to extensive labeling of the particulate fractions of the heart. The mitochondrial fraction possessed the largest portion of the bound $^{45}Ca^{2+}$ and was, in fact, the only fraction having more counts per mg protein than the whole homogenate. The specific activity of the mitochondrial Ca^{2+} pool was also the highest of the various fractions, indicating that mitochondrial calcium exchanges rapidly with extracellular Ca^{2+}. Correction of the values for radioactivity per mg protein for organelle distribution was found to give negative values for the microsomal fraction. This is obviously an artifact and may arise from the cumulative errors inherent in the several enzyme assays and ^{45}Ca measurements. Since the radioactivity of the mitochondrial fraction was much greater than that of the microsomal fraction, small errors in estimation of the composition of these fractions would be of less importance in the calculation of the mitochondrial $^{45}Ca^{2+}$ than the microsomal $^{45}Ca^{2+}$.

Calculation of the specific activity of the various fractions showed that this parameter was lowest in the microsomal fraction. Even if some of the Ca^{2+} in the sarcoplasmic reticulum has been lost during mincing and washing of the tissue the specific activity would not be affected. The fraction designated LM did have a high specific activity when corrections for contaminating mitochondria and microsomes were made. It is possible that this fraction represents a segment of the sarcoplasmic reticulum which interacts more directly with extracellular calcium.

In agreement with the observations of GROSSMAN AND FURCHGOTT[16] we found that epinephrine increased the exchange of intracellular and extracellular Ca^{2+}. On a

TABLE IV

SUBCELLULAR DISTRIBUTION OF ^{45}Ca IN RAT HEART

Data corrected as indicated in Table III.

Fraction	*Counts/min per mg protein*		*Specific activity (counts/min per μmoles Ca)*	
	Saline infusion	*Epinephrine infusion*	*Saline infusion*	*Epinephrine infusion*
WH	72.6 ± 6.6 (8)*	107.8 ± 10.9 (8)***	10700 ± 1050 (8)	18100 ± 1840 (8) §
R	68.6 ± 6.3 (9)	107.9 ± 9.3 (8)*	10100 ± 1330 (8)	18950 ± 1290 (8) §
M	176.4 ± 9.8 (8)	245.6 ± 18.8 (7)**, §	13210 ± 1050 (9)**	16300 ± 1580 (7)
LM	40.6 ± 2.8 (7)**	61.3 ± 6.4 (8)**, ***	8280 ± 1180 (9)	10300 ± 1600 (9)**
Mic	36.2 ± 4.6 (9)**	38.0 ± 5.4 (8)**	5590 ± 800 (8)**	6500 ± 1550 (8)**
S	18.4 ± 1.9 (9)**	27.1 ± 3.8 (9)**	4600 ± 460 (6)**	8900 ± 1200 (5)**, §
	Data adjusted for distribution of mitochondria and microsomes			
Residue	55	115	11440	25420
Mitochondria	225	319	15700	19700
Light mitochondria	50	48	17300	23200
Microsomes	−17	−40	2700	2760
Soluble	18	27	4590	9330

* Mean ± S.E. Number in parentheses = number of samples.
** $P < 0.01$ compared to WH.
*** $P < 0.02$ compared to the same fraction from saline-infused hearts.
§ $P < 0.01$ compared to the same fraction from saline-infused hearts.

protein basis, labeling was significantly increased in the WH, R, M, and LM fractions of the heart. The increase in the LM fraction was, however, calculated to be due to contaminating mitochondria. The specific activities of the WH, R, and S fractions were significantly increased. The specific activity of calcium in the mitochondrial fraction was greater in mitochondria from epinephrine-treated hearts than from control hearts although the difference was not statistically significant. Adjustment of the values for distribution of mitochondria and microsomes increased the difference between the values obtained from the two groups of hearts. This suggests that the rise in specific activity of mitochondrial calcium after epinephrine is, in fact, a real phenomenon. The differences in radioactivity of the R and LM fractions were also increased by these corrections. It was noted that the specific activity of the microsomal fraction was not altered by epinephrine.

DISCUSSION

There are many technical difficulties in the type of experimentation presented here. Most important is the possibility that redistribution of calcium occurs after the homogenization of the tissue. One cannot be entirely certain that no calcium movements take place during the preparation of the tissue fractions. However, we feel that such events have been minimized by the use of rotenone and oligomycin which are effective inhibitors of mitochondrial calcium uptake. In eight experiments we exposed the heart to rotenone and oligomycin by infusing a sucrose solution containing these inhibitors into the heart before we cooled and homogenized the tissue. The distribution of calcium between the different cell fractions was not significantly different from that found when the hearts were simply dropped into ice-cold sucrose containing oligomycin and rotenone.

The most important finding in our studies is the rapidity with which Ca^{2+} enter mitochondria in the perfused intact heart. When the heart was perfused with $^{45}Ca^{2+}$ for 10 sec, the accumulation of isotope was significantly greater in the presence than in the absence of epinephrine. These observations strongly support the view that mitochondria in the heart play a significant role in the movements of Ca^{2+} occurring during the cycle of contractions and relaxation of the myocardium.

This conclusion is also supported by the observation of PATRIARCA AND CARAFOLI[14] that a large percentage of $^{45}Ca^{2+}$ found in the heart after administration of the isotope *in vivo* is present in the mitochondria. The finding that oligomycin, a substance that effectively inhibits ATP-supported calcium uptake by mitochondria but not by microsomes[7], prevents relaxation of the intact heart[15] provides additional evidence for a role of mitochondria in regulating the force of contraction of the heart.

Our data indicate that that portion of sarcoplasmic reticulum found in the microsomal fraction is not in rapid equilibrium with extracellular calcium. If it does partake in calcium movements in the intact heart, it must do so with a pool relatively isolated from external calcium.

If mitochondria do indeed take part in the regulation of the concentration of free calcium at the site of the contractile proteins, extrusion of calcium from mitochondria must occur and be regulated by changes in concentration of appropriate cellular components. Little is known about the mechanisms involved in the efflux of calcium from mitochondria. Detailed information about these processes is essential

for an understanding of the possible role of mitochondria in regulating the intracellular calcium concentration.

The observation that administration of epinephrine to the perfused heart gives rise to an increased mitochondrial calcium exchange may be a reflection of the increased mechanical activity of the heart or it may be the result of a direct effect of the catecholamine or of cyclic 3′,5′-AMP on mitochondrial calcium transport.

ACKNOWLEDGMENTS

One of the authors (R.S.H.) gratefully acknowledges the support of the Pennsylvania Plan to Develop Scientists in Medical Research in the period during which this work was done. This research was supported by U.S. Public Health Service grant HE-01813 from the National Heart Institute.

REFERENCES

1 L. V. HEILBRUNN AND F. J. WIERCINSKI, *J. Cell. Comp. Physiol.*, 29 (1947) 15.
2 R. J. PODOLSKY AND L. L. CONSTANTIN, *Federation Proc.*, 23 (1964) 933.
3 W. HASSELBACH, *Prog. Biophys. Mol. Biol.*, 14 (1964) 167.
4 A. SANDOW, *Pharmacol. Rev.*, 17 (1965) 265.
5 A. M. KATZ, *Physiol. Rev.*, 50 (1970) 63.
6 B. FANBURG, R. M. FINKEL AND A. MARTONOSI, *J. Biol. Chem.*, 239 (1964) 2298.
7 B. FANBURG AND J. GERGELY, *J. Biol. Chem.*, 240 (1965) 2721.
8 A. M. KATZ AND C. T. REPKE, *Circulat. Res.*, 21 (1967) 153.
9 E. C. SLATER AND K. W. CLELAND, *Biochem. J.*, 55 (1963) 566.
10 G. P. BRIERLEY, E. MURER AND E. BACHMANN, *Arch. Biochem. Biophys.*, 109 (1964) 89.
11 B. CHANCE, *J. Biol. Chem.*, 240 (1965) 2729.
12 A. L. LEHNINGER, E. CARAFOLI AND C. S. ROSSI, *Advan. Enzymol.*, 30 (1968) 259.
13 M. C. O. FEHMERS, *Intracellular Ca^{2+} en de Werking van Hartspier*, Academic Thesis Univ. Amsterdam, Hollandia Offsetdrukkerij, Amsterdam, 1968.
14 P. PATRIARCA AND E. CARAFOLI, *J. Cell. Physiol.*, 72 (1968) 29.
15 R. S. HORN, R. LEVIN AND N. HAUGAARD, *Biochem. Pharmacol.*, 18 (1969) 503.
16 A. GROSSMAN AND R. F. FURCHGOTT, *J. Pharmacol. Exptl. Therapeut.*, 145 (1964) 162.
17 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
18 D. C. WHARTON AND A. TZAGOLOFF, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 245.
19 H. A. M. HULSMANS, *Biochim. Biophys. Acta*, 54 (1961) 1.
20 R. S. HORN, C. E. ARONSON, M. E. HESS AND N. HAUGAARD, *Biochem. Pharmacol.*, 16 (1967) 2109.
21 A. GROSSMAN AND R. F. FURCHGOTT, *J. Pharmacol. Exptl. Therapeut.*, 143 (1964) 107.