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Excitation-Contraction Coupling in Heart

III. Evidence against the Involvement of Adenosine Cyclic 3′, 5′-Monophosphate in Calcium Transport by Sarcotubular Vesicles of Canine Myocardium

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SUMMARY

Sarcotubular vesicles isolated from dog myocardium accumulated 2.51 ± 0.48 μmoles of calcium per milligram of protein in the presence of oxalate and hydrolyzed 18.39 ± 1.96 μmoles of ATP per milligram of protein in 30 min at 37°. Cyclic 3′, 5′-AMP (10^{-6}–10^{-5} M) influenced neither the rate nor the extent of calcium uptake and ATP hydrolysis. However, at higher concentrations (4×10^{-3} M), cyclic AMP reduced the ability of these membranes to accumulate calcium and hydrolyze ATP. Heart sarcotubular vesicles bound 66.54 ± 4.53 mμmoles of calcium per milligram of protein in the absence of oxalate and exchanged 15.82 mμmoles of calcium per milligram of protein in 2 min at 25°. Neither calcium binding nor the exchangeability of membrane-bound calcium was affected appreciably by cyclic AMP. About 60% of the bound calcium of the heart sarcotubular vesicles was released in the incubation medium in 10 min at 25° after the addition of EDTA. Cyclic AMP had no effect on the rate or the extent of calcium efflux in this system. These results do not indicate any action of cyclic AMP on calcium transport across the sarcotubular membranes of myocardium. Neither epinephrine nor glucagon influenced the ability of these vesicles to accumulate calcium in the absence or presence of oxalate. It is suggested that the postulated mechanism of increased calcium movement across cardiac sarcoplasmic reticulum due to an increased level of cyclic AMP under the influence of different inotropic interventions be considered with due caution.

INTRODUCTION

Rasmussen and Tenenhouse (1) have suggested that adenosine cyclic 3′,5′-monophosphate (cyclic AMP) is an important regulator of membrane permeability to calcium. This hypothesis has now been extended by Entman (2) for calcium transport across the sarcotubular vesicles of heart muscle. Likewise, other investigators have considered the involvement of cyclic AMP in calcium movements across the cardiac cell membranes (3–5). However, a great deal of controversy exists in the literature concerning the action of cyclic AMP on the calcium pump in the heart. On one hand, Entman *et al.* (6) have demonstrated an increase in calcium transport in the presence of cyclic AMP while, on the other, cyclic AMP or its dibutyryl derivative has been reported to exert no action on calcium uptake by heart sarcoplasmic reticulum and mitochondria (7, 8). Calcium

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uptake and release by the skeletal muscle sarcoplasmic reticulum were also not affected by cyclic AMP (9, 10). A critical review of the literature suggested that a detailed investigation concerning the interaction of cyclic AMP with sarcotubular vesicle would be necessary before any conclusion could be drawn in this regard. Therefore, in the present study, the influence of cyclic AMP over a wide range of concentration on various aspects of calcium transport, such as adsorption, translocation, and exchange by the heart sarcotubular vesicles, is investigated.

MATERIALS AND METHODS

Dog heart ventricles were freed of fat and connective tissue, thoroughly washed in 0.25 M sucrose containing 1 mM EDTA, pH 7.0, and homogenized in 6–7 volumes of a medium containing 10 mM $NaHCO_3$, 5 mM sodium azide, and 10 mM Tris-HCl, pH 6.8, in a Waring Blendor at 0–2° for 45 sec. The homogenate was filtered through four layers of gauze and centrifuged at 1000 × *g* for 20 min, and then at 10,000 × *g* for 20 min, to remove cell debris, myofibrils, nuclei, and mitochondria. The 10,000 × *g* supernatant fraction was again filtered through four layers of gauze and centrifuged at 37,000 × *g* for 45 min. The residue thus obtained was thoroughly washed, resuspended with a glass homogenizer in 0.6 M KCl, and centrifuged at 37,000 × *g* for 45 min. The sediment was suspended in a solution (50 mM KCl–20 mM Tris-HCl, pH 6.8) at a protein concentration of 3–5 mg/ml (11). This fraction is referred to as sarcotubular vesicles (sarcoplasmic reticulum). The data concerning biochemical marker enzyme activities, sensitivity to inhibitors of mitochondrial transport, and electron microscopic studies revealed negligible mitochondrial or myofibrillar contamination in this fraction. This method of isolating sarcotubular vesicles is essentially similar to that described by Harigaya and Schwartz (12).

Calcium uptake was measured by incubating sarcotubular membrane (0.050–0.10 mg/ml) in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 6.8), 4 mM ATP, 5 mM potassium oxalate, and 0.1 mM $^{45}CaCl_2$ at 37° in a total volume of 2.0 ml for various time intervals. The reaction was started by the addition of sarcotubular vesicles and stopped by passage through Millipore filters (0.45 μ), and the radioactivity in the protein-free filtrate was measured in a Packard liquid scintillation spectrometer. In some experiments sarcotubular vesicles were treated with cyclic AMP for 3 min before the reaction was started by the addition of calcium. ATP hydrolysis by the reticulum fraction was measured by estimating the amount of inorganic phosphate released in the same filtrate by the method of Fiske and Subba-Row (13). The results were corrected for nonenzymatic hydrolysis of ATP during the course of incubation.

Calcium binding was measured in the same incubation medium except that oxalate was omitted. Binding was carried out at room temperature, and the protein concentration in the reaction flask was tripled. The efflux of calcium from the loaded vesicles and calcium exchange were studied in the incubation medium used for calcium binding according to published procedures (14, 15). Cyclic AMP and ATP were purchased from P-L Biochemicals and Calbiochem. The purity of cyclic AMP was tested by chromatographic procedures, and this compound from both sources of supply exerted similar effects, reported under RESULTS. The pH of both cyclic AMP and ATP solutions was adjusted to 7.0 before use. All other reagents were analytical grade, and distilled, deionized water was used throughout the experimental procedure. The effects of cyclic AMP, epinephrine, and glucagon on calcium accumulation in the absence and presence of 2.5 mM oxalate were also studied, under experimental conditions similar to those described by Entman *et al.* (6).

RESULTS

Calcium uptake studies. Sarcotubular vesicles in the presence of oxalate and ATP accumulated 0.92 ± 0.19, 2.15 ± 0.31, and 2.51 ± 0.48 μmoles of calcium per milligram of protein at 3, 10, and 30 min, respectively (Table 1). These calcium uptake values are higher than those observed by Entman *et al.* (6) and Carsten (16) for

TABLE 1

Influence of various concentrations of cyclic AMP on calcium uptake by sarcotubular vesicles of canine myocardium

Results are the means ± standard errors of five experiments.

Cyclic AMP	Calcium uptake		
	3 min	10 min	30 min
M		*μmoles/mg protein*	
	0.92 ± 0.19	2.15 ± 0.31	2.51 ± 0.48
1×10^{-6}	0.90 ± 0.25	2.09 ± 0.50	2.39 ± 0.48
5×10^{-6}	0.91 ± 0.24	2.12 ± 0.45	2.46 ± 0.40
1×10^{-5}	0.90 ± 0.24	2.18 ± 0.46	2.47 ± 0.40
1×10^{-3}	0.62 ± 0.20[a]	1.80 ± 0.53	2.27 ± 0.45
4×10^{-3}	0.12 ± 0.03[a]	0.22 ± 0.10[a]	0.31 ± 0.18[a]

[a] Significant at $p < 0.05$.

cardiac muscle, but are in good agreement with those reported by other workers (12, 17, 18). Cyclic AMP in the concentration range of 10^{-6}–10^{-3} M failed to show any effect on the rate and extent of calcium accumulation up to 30 min of incubation, although 1 mM cyclic AMP decreased the initial rate. However, at high concentration (4×10^{-3} M), cyclic AMP reduced both the rate and extent of calcium uptake by dog heart sarcotubular vesicles ($p < 0.05$).

ATPase activity of sarcotubular vesicles. The results concerning ATP hydrolysis by sarcotubular (Mg^{++}-Ca^{++})-ATPase are shown in Table 2. These vesicles were observed to hydrolyze 18.39 ± 1.96 μmoles of ATP per milligram of protein in 30 min. On the basis of initial readings for 3 min, the rate of ATP hydrolysis was found to be 1.8 ± 0.2 μmoles/mg/min. Cyclic AMP (10^{-6}–10^{-3} M) did not show any influence on the initial rate or the extent of ATP hydrolysis. However, 4 mM cyclic AMP significantly reduced the ATPase activity of sarcotubular vesicles ($p < 0.01$).

Calcium-binding studies. It is likely that oxalate-augmented calcium transport by these vesicles is not a physiological phenomenon, and it is also possible that oxalate may mask any effect of cyclic AMP on the calcium transport process. It was thus attempted to investigate the influence of cyclic AMP on calcium binding in the absence of oxalate. The results of these experiments are presented in Table 3. Sarcoplasmic reticulum bound 33.96 ± 3.82, 50.55 ± 6.13, and 66.54 ± 4.53 mμmoles of Ca^{++} per milligram of protein at 30, 60, and 120 sec, respectively. Calcium binding by these membranes was absolutely ATP-dependent, since, in the absence of ATP, these vesicles bound about 6–8 mμmoles of calcium per milligram of protein. The magnitude of observed calcium binding is similar to that reported by other workers (12, 19). Cyclic AMP at all concentrations tested did not show any effect on ATP-dependent calcium binding, although 4 mM cyclic AMP reduced the binding at 120 sec. No effect of cyclic AMP was observed on calcium binding in the absence of ATP.

Efflux of calcium from loaded vesicles. The release of membrane-bound calcium into the incubation medium was studied in the presence and absence of cyclic AMP. The results are reported in Table 4. Only 13%, 17%, and 25% of the bound calcium was released into the medium at 15, 30, and 60 sec after the addition of EDTA. About 60% of the bound calcium was released in 10 min. Similar results were recently reported by Pretorius *et al.* (14). Cyclic AMP (10^{-5}–10^{-3} M) showed no influence on the rate or total release of calcium during 10 min. In this system, 3–5 mM caffeine was found to augment the rate of release from these membranes in a manner similar to that reported by other workers (14).

Exchange of membrane-bound calcium. One possible way in which cyclic AMP can influence membrane transport is by altering the calcium exchange at the membrane

TABLE 2

Effect of various concentrations of cyclic AMP on (Mg^{++}-Ca^{++})-ATPase activity of dog heart sarcoplasmic reticulum

Results are the means ± standard errors of five experiments.

Cyclic AMP	ATPase activity		
	3 min	10 min	30 min
M	*μmoles P_i released/mg protein*		
	5.40 ± 0.73	8.50 ± 0.96	18.4 ± 2.0
1×10^{-6}	5.73 ± 0.56	9.25 ± 0.58	18.4 ± 2.0
5×10^{-6}	4.69 ± 0.56	9.33 ± 0.34	18.7 ± 1.4
1×10^{-5}	5.33 ± 1.06	9.64 ± 0.58	18.4 ± 1.7
1×10^{-3}	6.13 ± 0.57	10.14 ± 0.81	18.4 ± 0.4
4×10^{-3}	3.98 ± 0.92[a]	6.35 ± 0.46[a]	13.2 ± 0.4

[a] Significant at $p < 0.05$.

TABLE 3

Binding of calcium by sarcotubular vesicles of dog heart in the absence and presence of different concentrations of cyclic AMP

Results are the means ± standard errors of five experiments.

Cyclic AMP	Calcium binding		
	30 sec	60 sec	120 sec
M	*mμmoles/mg protein*		
	34.0 ± 3.8	50.5 ± 6.1	66.5 ± 4.5
1×10^{-6}	36.2 ± 1.8	49.7 ± 1.9	62.8 ± 1.4
5×10^{-6}	33.1 ± 2.3	49.7 ± 4.1	66.3 ± 3.7
1×10^{-5}	33.3 ± 3.7	52.4 ± 3.6	64.2 ± 4.5
1×10^{-3}	33.0 ± 5.2	48.2 ± 2.9	58.4 ± 5.6
4×10^{-3}	31.1 ± 4.8	49.4 ± 3.3	50.6 ± 6.0[a]

[a] Significant at $p < 0.05$.

site. This possibility was tested by using heart sarcoplasmic reticular membranes, and the results are shown in Table 5. Vesicles were incubated with nonradioactive calcium for 5 min, and the exchange of bound calcium with radioactive calcium was determined. These membranes exchanged about 11, 12, and 15 mμmoles of calcium per milligram of protein at 30, 60, and 120 sec, respectively. This rate of calcium exchange was not appreciably influenced by the presence of various concentrations of cyclic AMP in the incubation medium.

Effect of cyclic AMP, epinephrine, and glucagon on calcium uptake and binding. Since the present results concerning the action of cyclic AMP conflict with those reported by Entman *et al.* (6), it was decided to study the effect of cyclic AMP on calcium uptake and binding under the experimental conditions outlined by these investigators. In addition, the effects of epinephrine and glucagon were also investigated. The concentrations of cyclic AMP, epinephrine, and glucagon are the same as employed by Entman *et al.* (6), and the results are shown in Tables 6 and 7. It was observed that cyclic AMP (5×10^{-6} M), epinephrine (1×10^{-6} M), and glucagon (3×10^{-8} M) did not influence the ability of sarcoplasmic reticulum to accumulate

TABLE 4

Efflux of radioactive Ca^{++} from loaded sarcotubular vesicles induced by EDTA in the absence and presence of cyclic AMP

Reticular membranes had bound 76 ± 2 mμmoles of calcium per milligram of protein before efflux was started. Each value is the mean ± standard error of five experiments.

Time after EDTA addition	Residual activity		
	Control	10^{-5} M cyclic AMP	10^{-3} M cyclic AMP
	%	%	%
15 sec	87.3 ± 1.9	87.0 ± 1.4	88.7 ± 2.3
30 sec	83.1 ± 3.3	82.6 ± 2.9	85.4 ± 1.7
1 min	75.7 ± 2.4	71.7 ± 2.7	78.2 ± 1.1
2 min	69.3 ± 2.4	66.2 ± 2.2	67.3 ± 0.5
4 min	57.9 ± 2.7	55.0 ± 2.7	54.0 ± 1.4
7 min	47.4 ± 2.0	46.2 ± 1.5	47.6 ± 2.1
10 min	39.8 ± 3.1	40.6 ± 2.1	38.8 ± 1.3

TABLE 5

Influence of various concentrations of cyclic AMP on ability of sarcotubular vesicles of dog heart to exchange membrane-bound calcium

Results are the averages of two experiments.

Cyclic AMP	Calcium exchange		
	30 sec	1 min	2 min
M	*mμmoles/mg protein*		
	11.5	11.8	14.6
1×10^{-6}	11.2	13.0	14.6
5×10^{-6}	11.5	13.1	14.6
1×10^{-5}	10.4	11.4	14.7
1×10^{-3}	10.1	11.4	12.4

calcium in the absence or presence of 2.5 mM oxalate. These experiments indicate that the differences between the results of this study and those reported by Entman *et al.* (6) may not have been due to the concentrations of reactants, temperature, and other conditions of incubation.

DISCUSSION

In this study we have failed to observe an increase in calcium transport across the sarcotubular vesicles of dog heart in the presence of cyclic AMP. This finding is in contrast to the observations made by Entman *et al.* (6), who observed an increase in calcium uptake, calcium binding, and calcium exchange by dog heart sarcotubular vesicles in the presence of cyclic AMP. Although the reason for such a discrepancy is not clear at present, a few points are worth mentioning in this regard. Entman *et al.* employed a preparation which was stored up to 3 days at 4°, while we used fresh membranes throughout the course of this study. We did not attempt to investigate the effect of cyclic AMP on an aged preparation, since we consider that the results obtained from such experiments are difficult to interpret. The control calcium-binding value (11.6 ± 1.1 mμmoles/mg of protein) reported by Entman *et al.* (6) is considerably lower than those obtained in this study and reported by other investigators (12, 19). Furthermore, their calcium uptake values in the presence of oxalate were about 0.45 μmole, in contrast to the 1.31 μmoles/mg of protein at 10 min obtained by us under similar conditions. It may be noted that our values for calcium uptake in the presence of 5 mM oxalate at 37° are 2.15 μmoles/mg of protein in 10 min. We believe that we have a better preparation of vesicles, which are capable of transporting more calcium per milligram of membrane protein. Earlier experiments performed by us, using rat heart at 25°, also failed to reveal any effect of cyclic AMP or its dibutyryl derivative (8). Although Entman *et al.* (6) used 5×10^{-7}–1×10^{-5} M cyclic AMP for calcium-binding studies, only one concentration of cyclic AMP (5×10^{-6} M) was reported for calcium uptake and exchange studies. On the other hand, we have employed a wide concentration range (10^{-6}–4×10^{-3} M)

TABLE 6

Calcium uptake by dog heart sarcotubular vesicles in the absence and presence of cyclic AMP, epinephrine, and glucagon

These experiments were performed under conditions similar to those described in detail by Entman *et al.* (6). The reaction mixture contained 0.12 M KCl, 5 mM MgATP, 10 mM histidine buffer (pH 7.0), 2.5 mM Tris oxalate, and 0.04–0.08 mg protein per milliliter. After a 10-min incubation at 25°, $^{45}CaCl_2$ was added (final concentration, 10^{-4} M), and the samples were taken at 10, 20, and 30 min. The values are the means ± standard errors of three experiments.

Addition	Calcium uptake		
	10 min	20 min	30 min
	μmoles/mg protein		
None	1.31 ± 0.05	1.90 ± 0.11	2.03 ± 0.18
Cyclic AMP (5×10^{-6} M)	1.29 ± 0.08	1.85 ± 0.09	1.99 ± 0.17
Epinephrine (1×10^{-6} M)	1.32 ± 0.07	1.91 ± 0.15	2.04 ± 0.20
Glucagon (3×10^{-6} M)	1.26 ± 0.06	1.80 ± 0.13	1.93 ± 0.13

TABLE 7

Calcium binding by dog heart sarcotubular vesicles in the absence and presence of cyclic AMP, epinephrine, and glucagon

These experiments were performed under conditions similar to those described by Entman *et al.* (6). The basic reaction medium contained 0.12 M KCl, 5 mM MgATP, 10 mM histidine buffer (pH 7.0), and 0.2–0.3 mg of protein per milliliter. After a 10-min incubation at 25°, $^{45}CaCl_2$ was added (final concentration, 2.5×10^{-5} M), and the samples were taken at 5 min. The values are the means ± standard errors of three experiments.

Addition	Calcium binding
	mμmoles/mg protein
None	24.9 ± 1.6
Cyclic AMP (5×10^{-6} M)	25.2 ± 1.3
Epinephrine (1×10^{-6} M)	24.2 ± 1.9
Glucagon (3×10^{-8} M)	22.6 ± 0.6

of cyclic AMP in this study. We find it difficult to resolve the contradiction between the results obtained in this study and those reported by Entman *et al.* (6), even though we have tested the effect of cyclic AMP on sarcotubular vesicles isolated from different species by various methods and under a number of experimental conditions. Thus the possibility of losing some cellular component necessary for cyclic AMP action in our preparation seems unlikely.

While this manuscript was in preparation, Shinebourne and White (20) showed that cyclic AMP in concentrations up to 1×10^{-4} M did not significantly increase calcium uptake by dog sarcoplasmic reticulum in either the absence or presence of theophylline, a potent inhibitor of phosphodiesterase. However, these workers observed that cyclic AMP in concentrations of 1×10^{-3} M markedly increased calcium accumulation by the reticulum. A critical analysis of the data presented by these workers reveals not only that their preparations were less active (0.45 μmole of calcium per milligram of protein in 10 min at 37°, but also that some of their preparations failed to accumulate calcium in the presence of ATP even up to 5 min of incubation [Table 3 of Shinebourne and White (20)]. Furthermore, in contrast to the results reported in this study and by Entman *et al.* (6), Shinebourne and White (20) have claimed that sarcoplasmic reticulum also accumulates calcium in the presence of cyclic AMP (10^{-3} M) when ATP is omitted from the incubation system. Since no data for control calcium binding in the absence of ATP were presented, these results are difficult to evaluate. It seems pertinent to mention that apart from the question of whether or not cyclic AMP is involved in sarcotubular calcium accumulation, the data obtained in our laboratory as well as by other investigators (6, 20) demonstrate some of the difficulties in interpreting results from the study of isolated organelles.

Cyclic AMP at 4×10^{-3} M was found to decrease calcium uptake as well as the ATPase activity of sarcotubular membranes. This observation may not be of any physiological significance, since the formation of cyclic AMP in such high concentrations in the heart seems unlikely. At any rate, the inability of cyclic AMP (10^{-6}–10^{-3} M) to influence calcium uptake, calcium binding, and calcium exchange is further supported by the observation that in the above concentration range this agent was without any effect on ATPase activity. Entman *et al.* (6) also failed to observe any effect of cyclic AMP (5×10^{-6} M) on the basic and calcium-stimulated ATPase activities of the dog heart sarcoplasmic reticulum. Furthermore, the ineffectiveness of cyclic AMP in altering the permeability of heart sarcotubular membranes to calcium is apparent from our experiments concerning calcium release. This observation is in agreement with results reported by Weber (10), using skeletal muscle sarcoplasmic reticulum. It may be pointed out that cyclic AMP has been shown to increase slightly the adsorption of calcium to artificial lipid membrane (21). Cyclic AMP was also suggested to enhance calcium transport in kidney tubules (22), but this view was not supported by Borle (23), who was unable to observe any effect of cyclic AMP or its dibutyryl derivative.

The data reported in this study do not reveal any effect of epinephrine or glucagon on calcium transport. Entman *et al.* (6, 15) have reported that calcium transport is

increased by epinephrine, glucagon and ouabain, although such a claim for these inotropic interventions is not supported by the observations made in numerous laboratories (5, 8, 19, 24–26). Some investigators could demonstrate an increase in the rate of calcium uptake only at a very high concentration of norepinephrine, and isoproterenol was not effective (27, 28). On the basis of results concerning the effect of cyclic AMP on calcium transport across heart sarcotubular vesicles, the presence of adenyl cyclase in this membrane, and the activation of adenyl cyclase by epinephrine and glucagon, it has been emphasized that the positive inotropic action of these agents is mediated through cyclic AMP by augmentation of the sarcotubular calcium pool (2, 6). Although this postulated role of cyclic AMP is attractive in the sense that it may serve as a molecular mechanism for explaining positive inotropy as due to several interventions, we consider that such a concept should be taken with reservations, since the data described in this paper concerning the action of cyclic AMP, epinephrine, and glucagon do not support the suggested mechanism. Furthermore, we have recently reported that the activation of adenyl cyclase by epinephrine is not associated with an increase in calcium uptake by heart sarcoplasmic reticulum (29). We have also observed that changes in adenyl cyclase activity and calcium transport ability of dog heart sarcotubular vesicles can be dissociated by prior treatment with phospholipase C or trypsin (30). Thus, in the light of the data at hand and some reports available in the literature, we believe that cyclic AMP may not be involved in calcium adsorption, exchangeability, and translocation in the sarcotubular vesicles of myocardium and hence may not constitute a mechanism for the positive inotropic action of epinephrine. Our present study, however, does not rule out the possible involvement of an adenyl cyclase–cyclic AMP system at the myocardial sarcolemma for the mechanism of epinephrine action described above. We also have no evidence to suggest whether or not cyclic AMP is involved in the positive inotropic action of catecholamines through metabolic processes or ionic fluxes across the cell membrane. It may be noted that changes in cyclic AMP levels in the heart have been dissociated from positive inotropy under certain experimental conditions (31–33).

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