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REGULATION OF CANINE HEART SARCOLEMMAL Ca^{2+}-PUMPING ATPase BY CYCLIC GMP

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Cyclic nucleotide modulation of the sarcoplasmic reticulum calcium pump has been recognized for some time. Little is known, however, of cyclic nucleotide effects on the sarcolemmal Ca^{2+}-pump. In sarcolemmal vesicles prepared from ventricular muscle by a recent technique (Jones, L.R., Maddock, S.W. and Besch, H.R. (1980) J. Biol. Chem. 255, 9971–9980) we have demonstrated via Millipore filtration that 10^{-8} M and 10^{-9} M cyclic GMP depressed the rate of ATP- and Mg^{2+}-dependent $^{45}Ca^{2+}$ uptake by 34% and 52%, respectively. Only at millimolar levels did cyclic AMP have any effect and the respective 5′-nucleotides had no effect at all. Parallel measurement of the associated (Ca^{2+} + Mg^{2+})-ATPase in the presence of either cyclic or 5′-nucleotides, however, revealed no concomitant depression in ATP hydrolysis. In another series of experiments, the cyclic GMP effect on $^{45}Ca^{2+}$ uptake was associated with a significant decrease in the pump V_{max}, and at the most effective concentration of cyclic GMP increased the apparent K_m for Ca^{2+}. These results suggest that cyclic GMP may depress ventricular Ca^{2+} efflux by decreasing the enzyme turnover and to a limited extent, decreasing pump affinity for Ca^{2+}. This supports a hypothesis whereby cyclic GMP might modulate both local biochemical and electrophysiological events by an effect on a discrete, regional pool of intracellular Ca^{2+}.

Introduction

The concentration of cytosolic free Ca^{2+} in the mammalian myocardial cell is maintained at below 10^{-6} M due, in a large part, to active Ca^{2+} extrusion by the Ca^{2+}-stimulated, Mg^{2+}-dependent ATPases of the sarcolemma and sarcoplasmic reticulum [1–3]. Both enzymes can respond to increased intracellular Ca^{2+} and have been shown to be regulated by Ca^{2+} + calmodulin-dependent mechanisms, whether directly as in the sarcolemmal (Ca^{2+} + Mg^{2+})-ATPase [4] or indirectly as in the sarcoplasmic reticular enzyme [5,6]. In addition, the sarcoplasmic reticulum Ca^{2+}-pump can respond to hormonal stimulation. The β-receptor-mediated abbreviated systole is obtained through a cyclic AMP-dependent phosphorylation of a 22 kDa protein component of the sarcoplasmic reticulum membrane, phospholamban, and augmented Ca^{2+} pumping into myocardial sarcoplasmic reticulum [6–8]. However, unlike its sarcoplasmic reticulum counterpart, little is known about the hormonal or cyclic nucleotide modulation of the sarcolemma (Ca^{2+} + Mg^{2+})-ATPase. In the present study we have investigated the effect of cyclic nucleotides on both the Ca^{2+}-pump and the (Ca^{2+} + Mg^{2+})-ATPase activity in highly purified heart sarcolemmal vesicles. There is some evidence from previous reports that cyclic AMP-dependent phosphorylation could increase Ca^{2+} uptake in cardiac membrane preparations [9–11], but there is some question as to the sarcolemmal purity and the possibility that some, if not all, of the changes noted were due to contaminating sarcoplasmic re-

ticulum. We now report that low concentrations of cyclic GMP, but not cyclic AMP, caused marked inhibition of the sarcolemmal Ca^{2+}-pump with little change in Ca^{2+}-dependent ATP hydrolysis, i.e., a cyclic GMP-dependent uncoupling of ATP hydrolysis and Ca^{2+} movement. This cyclic nucleotide-mediated event was associated with significant changes in pump V_{max}, and at one concentration of cGMP, an additional change in the apparent K_m of the pump for Ca^{2+}.

Materials

^{45}Ca (5–30 Ci/g Ca^{2+}) as $CaCl_2$ and [γ-^{32}P]ATP (200 Ci/mmol) as the sodium salt were both purchased from ICN (Montreal). Cyclic AMP, cyclic GMP, isobutylmethylxanthine, histone Type II S, dithiothreitol and Tris-ATP were all obtained from Sigma. The Ca^{2+} ionophore A23187 was obtained from Calbiochem (La Jolla, CA). All other chemicals were of reagent grade.

Methods

Sarcolemmal vesicles were prepared from 100 g of ventricular muscle obtained from mongrel dogs by the method of Jones et al. [12]. The vesicles were washed and suspended in 20 mM Tris-HCl/0.25 M sucrose (pH 7.4) and immediately frozen in liquid nitrogen at a protein concentration of approx. 600 $\mu g \cdot ml^{-1}$. The vesicles were stored at $-20°C$ and were used within 1 week of preparation.

$(Na^+ + K^+)$-ATPase and cyclic AMP- and cyclic GMP-dependent protein kinase activities were assayed as described by Post and Sen [13] and Grant et al. [14] respectively. In the cyclic GMP-dependent protein kinase assay, the concentration of Mg^{2+} was increased to 30 mM after Shoji et al. [15]. Protein content was determined as described by Lowry et al. [16] using bovine serum albumin as a standard.

The ^{45}Ca uptake studies were performed by means of Millipore filtration as described by Caroni and Carafoli [17] with the following minor changes. Tris-HCl buffer and Tris-ATP were utilized instead of Hepes buffer and dipotassium-ATP. Also, oligomycin addition, a mitochondrial inhibitor, was found unnecessary and was omitted. Studies on calcium uptake characterization, i.e., ATP and Mg^{2+} dependence, and reaction to A23187 and Na^+ addition were performed as described by Caroni and Carafoli [17].

The $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured in a medium identical to that described for the ^{45}Ca uptake studies, but with one exception. The Tris-ATP contained a minimum of 10^6 dpm $\cdot$ ml^{-1} [γ-^{32}P]ATP. The enzyme preparation was incubated for 10 min at 37°C. The reaction was stopped by the addition of perchloric acid and the hydrolyzed ^{32}P released was extracted as described by Post and Sen [13]. One ml of the butyl acetate extraction medium was then assessed for ^{32}P by means of liquid scintillation counting. Specific activity of the enzyme was measured in the presence of Mg^{2+} and was defined as the difference in ^{32}P released in the presence and absence of 150 μM Ca^{2+}. All assays were performed in duplicate.

In experiments designed to measure the Ca^{2+}-dependence of both the Ca^{2+} uptake and $(Ca^{2+} + Mg^{2+})$-ATPase activity, the free Ca^{2+} concentration was maintained at the required micromolar and submicromolar range by utilizing the buffering ligand ethylene glycol bis(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) after the method of Pershadsingh and McDonald [18].

Statistical analysis, when performed, was done according to the Student's t-test. The rate of ^{45}Ca uptake was obtained by means of linear regression analysis of the linear portion of the uptake curve. Double reciprocal plots were constructed to allow a rigorous calculation of apparent K_m and V_{max} values for the Ca^{2+}-dependence studies.

Results

Sarcolemma prepared as described appeared as irregular vesicles with diameters varying between 0.05 and 0.35 μm in electron micrographs (Fig. 1A). The micrographs also showed that the preparations were substantially free of contamination by mitochondria and other recognizable cell debris. Greater magnification showed that the vesicular membranes maintained the familiar bilayer structure (Fig. 1B).

Measurement of $(Na^+ + K^+)$-ATPase activity, a marker for plasma membranes, showed an enrichment of about 15-fold over the measured activ-

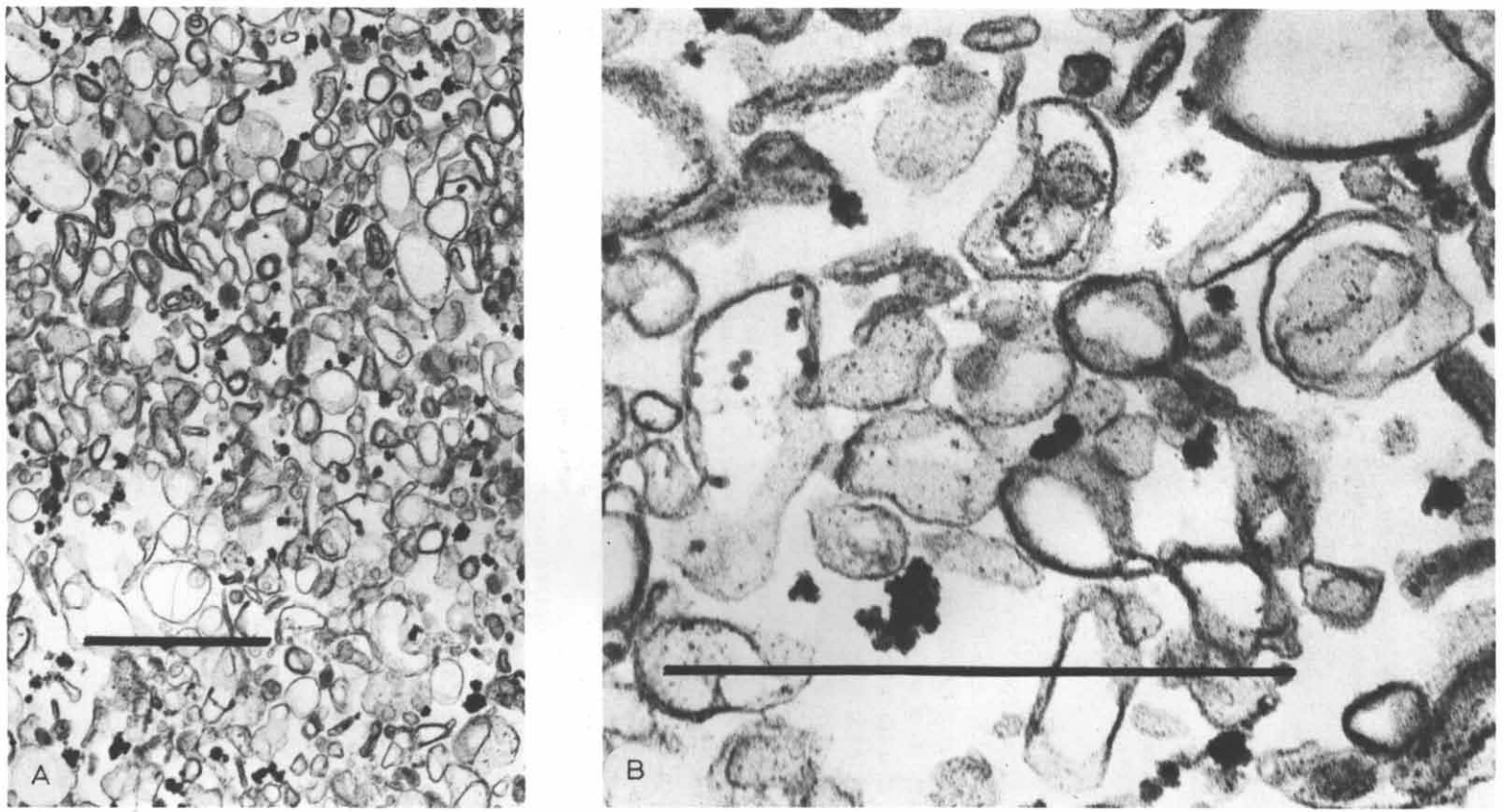

Fig. 1. Electron photomicrographs of heart sarcolemmal vesicular preparation used in these experiments. A, Magnification ×26028; B, magnification ×86184. Bars represent 1 μm.

TABLE I

MEMBRANE-BOUND CYCLIC GMP- AND CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY OF CANINE HEART SARCOLEMMAL VESICLES

Protein kinase activity was assayed using histone as a phosphate acceptor as described by Grant et al. [14]. The data are in pmol ^{32}P transferred/mg protein per min. Compared in the absence of both cyclic nucleotides, $P < 0.05$.

Nucleotide	Nucleotide concentration (M)		
	0	10^{-6}	*n*
i cGMP	580.4 ± 151.6	1185.4 ± 178.5	7
ii cAMP	632.9 ± 146.6	1314.9 ± 268.7	7

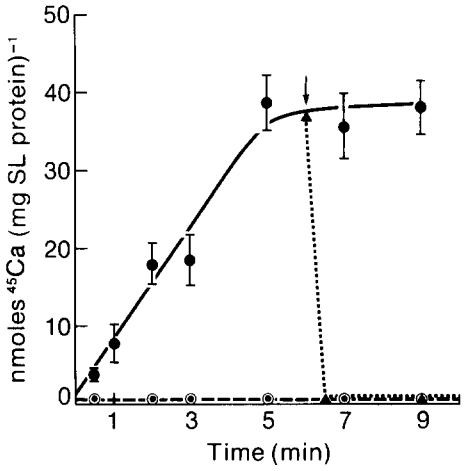

Fig. 2. ATP- and Mg^{2+}-dependent ^{45}Ca uptake profile in dog heart sarcolemma. Sarcolemmal vesicles (50–60 μg protein) were preincubated at 37°C in 1 ml of a medium containing 160 mM KCl/20 mM Tris-HCl/5 mM $MgCl_2$/1 mM ouabain (pH 7.4). After 5 min 150 nmol of ^{45}Ca were added; 2 min later the first aliquot was withdrawn (time zero averaged approx. 35 nmol ^{45}Ca/mg sarcolemma protein) and 1 mM Tris-ATP was added. Further 100 μl aliquots were withdrawn at the indicated points, filtered and washed through Millipore filters. The values were corrected for the ^{45}Ca found associated with the vesicles before ATP addition (time zero aliquot). ●———●, No supplementary addition (control ± S.E.), $n = 6$: Four representative experiments; ⊙------⊙, no ATP or no Mg^{2+}; ▲·····▲, at the arrow 5 μM A23187 or 30 mM NaCl (final concentrations) were added. The slope of the linear regression analysis of the initial 5 min uptake was defined as 'Ca^{2+} uptake rate' (nmol ^{45}Ca/mg protein per min).

ity in the crude homogenate. $(Na^+ + K^+)$-ATPase activity averaged 35.2 ± 3.6 μmol P_i released/mg protein per h in eight sarcolemmal preparations. Treatment of the membranes with 0.3 mg sodium dodecyl sulfate/ml, a method to unmask latent enzyme sites, increased the activity of one preparation from 26.7 to 79.0 μmol P_i released/mg protein per h.

Table I shows that, in addition to $(Na^+ + K^+)$-ATPase, the preparation contained significant amounts of membrane-bound cyclic AMP- and cyclic GMP-dependent protein kinase activity, enzymes through which the effects of both cyclic nucleotides are thought to be mediated in most cases.

The uptake profile of the ^{45}Ca uptake experiments is shown in Fig. 2. Uptake was linear during the initial 5 min period and then plateaued. The slope of this initial 5 min was defined as 'uptake rate' and was calculated by linear regression analysis. The omission of ATP or Mg^{2+} from the uptake medium resulted in no Ca^{2+} uptake. As well, the addition of either 30 mM Na^+ (as NaCl) or 5 μM A23187, a Ca^{2+} ionophore, resulted in complete loss of intravesicular Ca^{2+}. Furthermore,

TABLE II

EFFECT OF CYCLIC GMP ON Ca^{2+} UPTAKE RATE INTO HEART SARCOLEMMAL VESICLES

(i) No additional agents; (ii) uptake in the presence of isobutylmethylxanthine (1.0 mM) as well as cyclic GMP. Data represent mean ± S.E., where uptake rate is defined as the slope of the linear regression line through the initial 5 min uptake period. Units are nmol ^{45}Ca/mg protein per min, i.e., uptake rate. [a] $P < 0.05$ as compared to the proper control (zero cyclic GMP).

	cGMP concentration (M)					
	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}	*n*
i	8.08 ± 1.70	6.69 ± 1.22	4.19 ± 0.47 [a]	5.33 ± 1.08 [a]	5.57 ± 0.91	6
ii	7.26 ± 1.31	5.58 ± 0.80 [a]	4.79 ± 0.71 [a]	5.56 ± 1.13 [a]		6

TABLE III

EFFECT OF CYCLIC AMP (i), 5'AMP (ii) AND 5'GMP (iii) ON Ca^{2+} UPTAKE RATE INTO HEART SARCOLEMMAL VESICLES

Data in nmol ^{45}Ca/mg protein per min, i.e., uptake rate, represents mean ± S.E. for experiments with n as shown. In (i) it should be noted the concentration range tested was much greater than that for (ii) and (iii). The experiments were performed at different times and on different sarcolemma preparations and accounts for the slightly different control values.

Nucleotide	Nucleotide concentration (M)					
	0	10^{-9}	10^{-7}	10^{-5}	10^{-3}	n
i cAMP	7.11 ± 1.04	8.01 ± 0.63	7.12 ± 0.42	6.02 ± 0.17	5.24 ± 0.50 [a]	3
	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}	
ii 5'AMP	4.93 ± 1.29	5.22 ± 1.87	4.58 ± 1.29	4.50 ± 1.39	5.00 ± 1.32	4
iii 5'GMP	6.91 ± 2.50	5.91 ± 2.17	5.51 ± 1.35	4.79 ± 0.77	4.62 ± 1.25	4

while oxalate addition did not stimulate uptake (data not shown), low (10^{-6} M) concentrations of chlorpromazine, thought to antagonize calmodulin activation of (Ca^{2+} + Mg^{2+})-ATPase [27], depressed it (data not shown). This information taken together suggests that the vesicles isolated are indeed of sarcolemmal origin, that they 'accumulate' Ca^{2+} rather than simply 'binding' Ca^{2+} and that the accumulation is partly dependent upon calmodulin.

The addition of cyclic GMP resulted in a significant depression in ^{45}Ca uptake rate. As shown in Table II, 10^{-8} M and 10^{-9} M cyclic GMP depressed ^{45}Ca uptake by 34% and 52%, respectively. If the phosphodiesterase inhibitor isobutylmethylxanthine was added to the assay with the cyclic GMP, the decrease in ^{45}Ca uptake rate was observed with a concentration as low as 10^{-10} M cyclic GMP (Table II).

Table III confirms that the effect is indeed specific for cyclic GMP. Cyclic AMP had no effect on ^{45}Ca uptake rate except at a concentration of 1 mM which, in all probability, is unphysiological. In addition, 5'AMP and 5'GMP could not alter ^{45}Ca uptake rates.

To ensure that the (Ca^{2+} + Mg^{2+})-ATPase activity that was measured in this sarcolemmal pre-

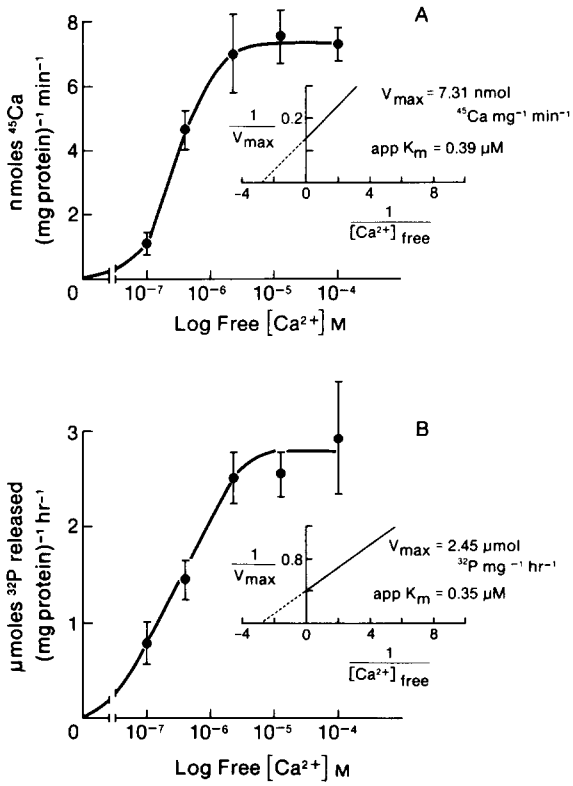

Fig. 3. Ca^{2+}-dependence of both ATP-driven Ca^{2+} uptake (A) and of (Ca^{2+} + Mg^{2+})-dependent ATPase activity (B) of heart sarcolemmal vesicles. ^{45}Ca uptake and (Ca^{2+} + Mg^{2+})-ATPase activity were measured as described in Methods, based on work by Caroni and Carafoli [17]. Free Ca^{2+} concentration was maintained from 100 μM to 0.1 μM by EGTA as described by Pershadsingh and McDonald [18]. Data are shown as mean ± S.E. for three separate experiments in each case. Insets: double-reciprocal plot of same data; A, nmol ^{45}Ca uptake/mg protein per min and B, μmol P_i released/mg protein per h vs. activation due to 100, 25, 5, 0.75 and 0.1 μM free Ca^{2+}.

TABLE IV

EFFECT OF CYCLIC AND 5'-GUANOSINE AND ADENOSINE NUCLEOTIDES ON HEART SARCOLEMMA (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

Sarcolemmal vesicles (20–30 μg protein) were incubated for 10 min in 1 ml of a medium comprising 160 mM KCl/20 mM Tris-HCl/5 mM $MgCl_2$/1 mM ouabain (pH 7.4). The reaction was started with the addition of 1 mM Tris-ATP containing a minimum of 10^6 dpm/ml [γ-^{32}P]ATP. Specific activity of the enzyme was defined as the difference between P_i released in the presence and in the absence of 150 μM $CaCl_2$. Data are in μmol P_i released/mg protein per h and represent mean ± S.E. [a] $P < 0.05$ as compared to the respective control.

Nucleotide	Nucleotide concentration (M)					
	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}	n
i cGMP	3.13 ± 0.64	3.77 ± 0.80 [a]	3.67 ± 0.92	4.30 ± 0.70	3.36 ± 1.01	6
ii cAMP	3.13 ± 0.64	3.70 ± 0.80	3.71 ± 0.91	3.87 ± 0.95	3.14 ± 0.46	6
iii 5'GMP	3.22 ± 0.48	4.36 ± 0.54	4.08 ± 0.33 [a]	3.74 ± 0.49	3.64 ± 0.61	5
iv 5'AMP	3.22 ± 0.48	4.08 ± 0.53	3.54 ± 0.38	3.74 ± 0.56	4.24 ± 0.53 [a]	5

paration was the biochemical manifestation of the associated ^{45}Ca uptake, the Ca^{2+} dependence of both the Ca^{2+} uptake and (Ca^{2+} + Mg^{2+})-ATPase was measured in three separate experiments. As Fig. 3 shows, the apparent K_m values, 0.39 μM Ca^{2+}_{free} for the ^{45}Ca uptake (Fig. 3A) and 0.35 μM Ca^{2+}_{free} for the (Ca^{2+} + Mg^{2+})-ATPase (Fig. 3B) were separated by only 0.04 μM Ca^{2+}. These data support our hypothesis that these two parameters are two separate measurements of the same enzyme system.

Table IV illustrates the results of five or six experiments measuring the specific activity of the (Ca^{2+} + Mg^{2+})-dependent ATPase associated with the sarcolemmal vesicular preparation, in the presence of cyclic GMP, cyclic AMP, 5'GMP and 5'AMP. It is obvious that although cyclic GMP depressed ^{45}Ca uptake rates there was no concomitant depression in (Ca^{2+} + Mg^{2+})-ATPase activ-

TABLE V

EFFECT OF 10^{-8} M AND 10^{-9} M CYCLIC GMP ON THE APPARENT K_m AND V_{max} OF THE HEART SARCOLEMMAL ATP-DRIVEN Ca^{2+} UPTAKE

Data represents mean ± S.E. for four determinations derived from double-reciprocal plots of the Ca^{2+}-dependence of ^{45}Ca uptake experiments as described in Fig. 3A and as shown in part in Fig. 4. [a] $P < 0.05$ as compared to control; [b] $P < 0.005$ as compared to control.

Additions	Apparent K_m (μM Ca^{2+}_{free})	V_{max} (nmol Ca^{2+}/mg protein per min)
None	0.509 ± 0.104	9.88 ± 1.27
10^{-8} M cGMP	0.543 ± 0.056	8.99 ± 1.17 [a]
10^{-9} M cGMP	0.636 ± 0.137 [a]	7.89 ± 1.32 [b]

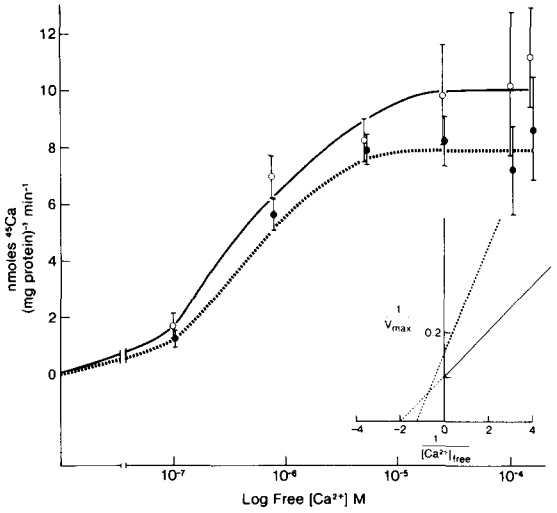

Fig. 4. The effect of 10^{-9} M cyclic GMP on the Ca^{2+}-dependence of ATP-driven Ca^{2+}-uptake in heart sarcolemmal vesicles. Methodology is as described in Methods and in Fig. 3, except that Ca^{2+}-activation has been extended to include 150 μM free Ca^{2+}. Data are shown as mean ± S.E. for four separate experiments in both cases. Inset: Double-reciprocal plot of same data. O ——— O, no supplementary additions; ●------●, 10^{-9} M cyclic GMP.

ity. Indeed, in a number of cases there is a small but significant increase in specific activity. However, since this effect was not either concentration- or compound-dependent, it would seem highly unlikely that it represents a physiological phenomenon.

The Ca^{2+} dependence of the ^{45}Ca uptake was measured in the presence of 10^{-8} M and 10^{-9} M cyclic GMP, in order that we might determine the mechanism whereby the cyclic nucleotide depresses Ca^{2+} transport. The results for this series are shown in Fig. 4 and in Table V. The data from these experiments show that in the presence of cyclic GMP, at concentrations effective in decreasing the rate of ^{45}Ca uptake, there was a significant and parallel depression in the V_{max} of the pump. As Fig. 4 shows, at 150 μM free Ca^{2+}, for example, 10^{-9} M cyclic GMP depresses ^{45}Ca uptake by approx. 23%. This is associated with a 20% decrease in pump V_{max}. A similar effect was seen at 10^{-8} M cyclic GMP (data not shown).

In addition to the pronounced effect on V_{max}, cyclic GMP at 10^{-9} M also significantly increased the apparent K_m of the enzyme for Ca^{2+}. The effect at 10^{-8} M, while not significant, showed a tendency to increase the K_m as well.

Discussion

The cyclic nucleotide modulation of several transport enzyme systems has become increasingly clear in the last decade. The most ubiquitous and clearly understood transport enzyme regulated by a cyclic nucleotide mechanism is the plasma membrane-bound $(Na^+ + K^+)$-ATPase. Cyclic AMP has recently been demonstrated to inhibit $(Na^+ + K^+)$-ATPase activity in membrane preparations from kidney [19], liver [20], heart [21] and brain [22]. Conversely, the same nucleotide has been hypothesized to control smooth muscle contractility via $(Na^+ + K^+)$-ATPase stimulation [23]. Cholinergic agonists have been shown to increase the Na^+-pump activity in avian salt glands and in rat submandibular glands, an effect that appears to be mediated via cyclic GMP [24].

Ca^{2+}-dependent ATPases, especially those associated with the myocardium, are another group of transport enzymes that are regulated by cyclic nucleotides. Perhaps the best example is the cyclic AMP-dependent stimulation of the sarcoplasmic reticular Ca^{2+}-pump in response to β-adrenergic agents, resulting in an increased rate of Ca^{2+} removal from the cytosol and a shortened cardiac systole [6–8]. In addition, a Ca^{2+} + calmodulin-dependent regulation of the sarcoplasmic reticulum Ca^{2+}-pump has also recently been demonstrated [5,6]. With the relatively new development of techniques that make possible the unequivocal separation of sarcolemma and sarcoplasmic reticulum isolated from myocardial cells [12,25,26], a highly specific $(Ca^{2+} + Mg^{2+})$-ATPase has been shown to exist in the sarcolemma as well [4,17,25,26]. This enzyme has subsequently also been demonstrated to be stimulated by a Ca^{2+} + calmodulin-dependent mechanism [4], identical to the phenomenon identified for a similar enzyme in mammalian erythrocytes [27]. However, little, if anything, is known concerning a cyclic nucleotide modulation of the sarcolemmal Ca^{2+}-pump.

In this investigation, evidence has been presented that suggests that cyclic GMP inhibits the Mg^{2+}-dependent Ca^{2+}-pump in canine heart sarcolemma. In sarcolemmal vesicles prepared substantially free of contamination, submicromolar levels of cyclic GMP inhibited the rate of ATP- and Mg^{2+}-dependent Ca^{2+} uptake. This transport is into inside-out vesicles prepared as described and simulates an in vivo Ca^{2+} efflux mechanism [4,17]. In addition, the concentrations of cyclic GMP used are low and reflect levels that might exist intracellularly [28]. The cyclic GMP effect was specific, as is shown by the lack of cyclic AMP efficacy, even at a wider concentration range, and of the 5′-nucleotides. Indeed, the evidence that the phosphodiesterase inhibitor isobutylmethylxanthine supported inhibition and increased the significance to 10^{-10} M cyclic GMP, further supports the conclusion that it is a specific GMP-mediated effect.

The finding that the ATP hydrolysis does not change in parallel with Ca^{2+} uptake is, at first glance, puzzling. The $(Ca^{2+} + Mg^{2+})$-ATPase located in various cell membranes is thought to serve the dual function of energy transduction and ion translocation in supporting the active transport of Ca^{2+}, i.e., it is the biochemical manifestation of the Ca^{2+}-pump. Extensive studies of the Ca^{2+} transport system of red blood cells and

skeletal muscle sarcoplasmic reticulum have shown a tight coupling between Ca^{2+} translocation and ATP hydrolysis, with a stoichiometry of 2 mol Ca^{2+} transported/mol ATP hydrolyzed [29,30]. However, in the present study this stoichiometry could not be obtained (estimated ratio approx. 0.18 mol Ca^{2+}/mol ATP). This lack of agreement with theoretical efficiency can be found throughout the literature, specifically when examining the data available concerning the Ca^{2+} pump from the plasma membrane of various tissues [4,17,25,31,32]. It may well be that, for some as yet unknown reason, the Ca^{2+} transport across the plasmalemma is already dissociated from hydrolysis, reflecting a somewhat more inefficient mechanism.

Secondly, there are numerous reports in which altered Ca^{2+} transport is not reflected in parallel changes in ATP hydrolysis. Work done on both sarcoplasmic reticulum and sarcolemma vesicles has shown that whereas Ca^{2+} uptake into aged heart sarcoplasmic reticulum was depressed and the same uptake into sarcolemma vesicles was enhanced as compared to the young rat, there was no difference in the $(Ca^{2+} + Mg^{2+})$-ATPase activity in any of the membranes [33]. Secondly, a marked inhibition of Ca^{2+} transport was seen after binding of anti-ATPase antibodies to sarcoplasmic reticulum membranes without causing a significant change in ATPase activity or Ca^{2+} permeability [34]. Similarly, following tryptic cleavage of a specific region of the ATPase molecule from sarcoplasmic reticulum, ATP hydrolysis remained unchanged while Ca^{2+} transport was wholly abolished [35]. In two different skeletal muscle sarcoplasmic reticulum preparations, while palmitic acid addition augmented Ca^{2+} sequestration and arachidonic, linoleic and oleic acids depressed Ca^{2+} uptake, all the long-chain fatty acids stimulated the associated ATPase activity [36,37]. Work in reconstituted enzyme systems reflects similar changes. For example, a number of reports have shown that Ca^{2+} translocation in reconstituted enzyme systems can be altered by membrane phospholipids and various proteolipids without invoking a change in ATPase activity [29,38]. Finally, studies from the laboratories of Racker and of Katz have shown that in reconstituted Ca^{2+}-pump vesicles of sarcoplasmic reticulum, augmented ATPase activity was associated with diminished Ca^{2+} transport rates, a difference that could not be accounted for by increased Ca^{2+} permeability of the reconstituted vesicles [38,39].

Thus it seems conceivable that the decreased rate of ^{45}Ca uptake seen after the addition of cyclic GMP may reflect further impairment in coupling of ATP hydrolysis to Ca^{2+} translocation. The exact mechanism of the cyclic GMP-dependent regulation of the Ca^{2+} pump is at present unclear and further work aimed at elucidating it is underway in this laboratory. Although there are data indicating that cyclic GMP can act directly to modify some enzyme activities [40], most evidence concerning cyclic nucleotide modulation of transport enzymes occurs through the appropriate protein kinase [41]. Recent work from Carafoli's group has indicated that a cyclic AMP-dependent protein kinase phosphorylation process might be involved in stimulating the cardiac sarcolemma Ca^{2+}-pumping ATPase [42]. This regulation was only seen, however, after extensive initial dephosphorylation of the membranes, and it is probably the reason cyclic AMP had no effect in our study. It is tempting to speculate that the modulation demonstrated in this study is dependent upon a cyclic GMP-dependent phosphorylation as the sarcolemma preparation used here contained significant levels of the appropriate kinase. Whatever the exact mechanism, whether direct or via a cyclic GMP-dependent protein kinase phosphorylation of the enzyme or a nearby substrate protein, our data indicate that the cyclic nucleotide ultimately decreased the turnover rate of the Ca^{2+} transport enzyme. In addition, cyclic GMP caused a significant decrease in enzyme affinity for Ca^{2+} at its most effective concentration in depressing ^{45}Ca uptake rate. At $1 \cdot 10^{-8}$ M cyclic GMP, while the change in K_m was not significant, it also showed a tendency to increase. In our experiments, at the concentration of Ca^{2+} used in most cases, the inhibition of ^{45}Ca uptake rate would most certainly have to be associated with the decrease in V_{max}. However, the change in enzyme affinity cannot be discounted. At levels of calcium of 1 μM or lower, a change in the apparent K_m of this magnitude would most certainly contribute to enzyme regulation. This inhibition of the sarcolemmal Ca^{2+}-pump, i.e., where there are decreases in both

enzyme turnover and affinity, is an example of a mixed type of enzyme inhibition.

The impairment in Ca^{2+} transport observed in the sarcoplasmic reticulum from aged hearts by Narayanan [33] was hypothesized to be associated with a decreased conformational response of the ATPase due to age-related changes in membrane composition. A similar impairment, leading to both a decreased enzyme turnover, and a decrease in Ca^{2+} affinity, might be caused by a cyclic GMP-dependent event at, or near, the sarcolemmal ATPase molecule. Although the reports often conflict, the calmodulin stimulation of various plasma membrane Ca^{2+}-pumps has been shown to involve significant increases in the enzyme turnover and/or increases in affinity for Ca^{2+} [4,43–45]. It is therefore conceivable that a cyclic GMP-dependent event might decrease the calmodulin-enzyme interaction, resulting in a decrease in the V_{max} or an increase in the apparent K_m.

Cholinergic agents and cyclic GMP have been most often associated with reducing the contractile force in most heart preparations. It is unlikely, however, that a cyclic GMP-dependent modulation of the sarcolemma Ca^{2+} pump would be associated with this phenomenon. Firstly, the sarcolemma used in these experiments is derived from ventricular muscle, and it is known that cholinergic agents have little effect on the force of contraction in mammalian ventricle [46,47]. Secondly, it is not likely that the $(Ca^{2+} + Mg^{2+})$-ATPase of the sarcolemma can modulate Ca^{2+} levels in quantities sufficient to effect contractility. The enzyme makes up, at most, only 1% of sarcolemmal protein [4]. In addition, the Ca^{2+}-pump has a very high affinity for Ca^{2+} and a relatively low maximal pumping rate and has been hypothesized to be responsible only for the maintenance of low Ca^{2+} levels during diastole [4,25]. Since changes in cyclic GMP concentration have been possibly associated with only small local effector pools [48] and due to the nature of the Ca^{2+}-pump itself, it seems likely that this enzyme alters the Ca^{2+} level only in discrete intracellular compartments and by this means the cyclic GMP-dependent inhibition of activity may modulate only local biochemical and electrophysiological events. By changing Ca^{2+} fluxes through the sarcolemma, cyclic GMP may increase the K^+ conductance associated with cholinergic stimulation in the myocardium [49]. Secondly, by increasing the $[Ca^{2+}]$ in intracellular pools, the depression in enzyme activity may regulation various biochemical pathways, especially 3′,5′-cyclic nucleotide metabolism, by activating the Ca^{2+}-binding modulator protein, calmodulin. It may be by this means that choline esters and cyclic GMP exert part of their 'antiadrenergic' antagonism. However, controversy surrounds the role of cyclic GMP in the myocardium. Recent work has suggested not only a dissociation between negative inotropy and increases in cyclic GMP, but a concomitant separation in cyclic GMP elevations and electrophysiological and metabolic effects of acetylcholine [50]. Indeed, before assigning a definitive role for this nucleotide control mechanism, the role of cyclic GMP in cardiac function awaits further clarification.

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References

1 Baker, P.F. (1978) Ann. N.Y. Acad. Sci. 307, 250–268
2 DiPolo, R. and Beauge, L. (1980) Cell Calcium 1, 147–169
3 Waisman, D.M., Gimble, J.M., Goodman, D.B.P. and Rasmussen, H. (1981) J. Biol. Chem. 256, 409–414
4 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263–3270
5 Katz, S. and Remtulla, M.A. (1978) Biochem. Biophys. Res. Commun. 83, 1373–1379
6 LePeuch, C.J., Haiech, J. and Demaille, J.G. (1979) Biochem. 18, 5150–5157
7 Hicks, M.J., Shigekawa, M. and Katz, A.M. (1979) Circ. Res. 44, 384–391
8 Kranias, E.G., Mandel, F., Wang, T. and Schwartz, A. (1980) Biochem. 19, 5434–5439
9 Will, H., Schirpke, B. and Wollenberger, A. (1973) Acta Biol. Med. Germ. 31, K45–52
10 Hui, C-W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415–427

11 Sulakhe, P.V., Leung, N.L. and St. Louis, P.J. (1976) Can. J. Biochem. 54, 438–445
12 Jones, L.R., Maddock, S.W. and Besch, H.R. (1980) J. Biol. Chem. 255, 9971–9980
13 Post, R.L. and Sen, A.K. (1967) Methods Enzymol. 10, 762–768
14 Grant, W., Breithaupt, T. and Cunningham, E. (1979) J. Biol. Chem. 254, 5726–5733
15 Shoji, M., Patrick, J.G., Davis, C.W. and Kuo, J.F. (1977) Biochem. J. 161, 213–221
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Caroni, P. and Carafoli, E. (1980) Nature 283, 765–767
18 Pershadsingh, H.A. and McDonald, J.M. (1980) J. Biol. Chem. 255, 4087–4093
19 Braughler, J. and Corder, C. (1978) Biochim. Biophys. Acta 424, 455–465
20 Barnabei, O., Luly, P., Tomasi, V., Trevisani, A. and Tria, E. (1973) in Advances in Enzyme Regulation (Weber, G., ed.), Vol. 2, pp. 274–290, Pergamon Press, New York
21 Limas, C., Notargiacomo, V. and Cohn, J. (1973) Cardiovasc. Res. 7, 477–481
22 Lingham, R.B. and Sen, A.K. (1982) Biochim. Biophys. Acta 688, 475–485
23 Scheid, C.R., Honeyman, T.W. and Fay, F.S. (1979) Nature 277, 32–36
24 Stewart, D.J. and Sen, A.K. (1981) Am. J. Physiol. 240, C207–C214
25 Morcos, N.C. and Drummond, G.I. (1980) Biochim. Biophys. Acta 598, 27–39
26 Trumble, W.R., Sutko, J.L. and Reeves, J.P. (1980) Life Sci. 27, 207–214
27 Raess, B. and Vincenzi, F.F. (1980) Mol. Pharmacol. 18, 253–258
28 Kuo, J.F., Lee, T.-P., Reyes, P.L., Walton, K.G., Donnelly, T.E., Jr, and Greengard, P. (1972) J. Biol. Chem. 247, 16–22
29 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1–79
30 Till, U., Petermann, H., Wenz, I. and Arese, P. (1981) Cell Calcium 2, 493–507
31 Lichtman, A.H., Segal, G.B. and Lichtman, M.A. (1981) J. Biol. Chem. 256, 6148–6154
32 McDonald, J.M., Pershadsingh, H.A., Kiechle, F.L. and Jarett, L. (1981) Biochem. Biophys. Res. Commun. 100, 857–864
33 Narayanan, N. (1981) Biochim. Biophys. Acta 678, 442–459
34 Sumida, M. and Sasaki, S. (1975) J. Biochem. 78, 757–762
35 Shamoo, A.E., Scott, T.L. and Ryan, T.E. (1977) J. Supramol. Struct. 6, 345–353
36 Messineo, F.C., Favreau, C. and Katz, A.M. (1981) Circulation 64, IV-163
37 Cheah, A.M. (1981) Biochim. Biophys. Acta 648, 113–119
38 Racker, E. (1980) Fed. Proc. 39, 2422–2426
39 Repke, D.I., Spivak, J.C. and Katz, A.M. (1976) J. Biol. Chem. 251, 3169–3175
40 Beavo, J.A., Hardman, J.G. and Sutherland, E.W. (1971) J. Biol. Chem. 246, 3841–3846
41 Kuo, J.F., Shoji, M. and Kuo, W.-N. (1978) Annu. Rev. Pharmacol. Toxicol. 18, 341–355
42 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 9371–9373
43 Lynch, T.J. and Cheung, W.Y. (1979) Arch. Biochem. Biophys. 194, 165–170
44 Niggli, V., Adjunyah, E.S., Penniston, J.T. and Carafoli, E. (1981) J. Biol. Chem. 256, 395–401
45 Pershadsingh, H.A., Landt, M. and McDonald, J.M. (1980) J. Biol. Chem. 255, 8983–8986
46 Watanabe, A.M. and Besch, H.R. (1975) Circ. Res. 37, 309–317
47 Schwegler, M., Reutter, K., Sieber, G. and Jacob, R. (1976) Basic Res. Cardiol. 71, 407–419.
48 Mirro, M.J., Bailey, J.C. and Watanabe, A.M. (1979) Circ. Res. 45, 225–233.
49 Trautwein, W. and Dudel, J. (1958) Pfluegers Arch. Ges. Physiol. 266, 324–334
50 Linden, J. and Brooker, G. (1979) Biochem. Pharmacol. 28, 3351–3360