EFFECT OF 3'5'-AMP ON CALCIUM-ACTIVATED ATPASE IN RAT HEART SARCOLEMMA

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SUMMARY

A recently described Ca^{++}-stimulated ATPase in the sarcolemmal fraction of rat heart exhibited a dose-dependent inhibition by 3'5'-AMP over the range of 10^{-8} - 10^{-4}M. Already at 3.3 x 10^{-8}M the effect was highly significant. A maximal inhibition of 33.8% was observed at 3.3 x 10^{-5}M 3'5'-AMP, while 5'-AMP was ineffective. The cyclic nucleotide had no effect upon Mg^{++} - or Na^{+}, K^{+} stimulated ATP hydrolysis in the same sarcolemmal preparation. The observation suggests a link between β-adrenergic stimulation of adenyl cyclase and active Ca^{++} transport mediated by cyclic AMP.

INTRODUCTION

Energy dependent uptake of calcium into guinea pig atria (1) and the recent observation of a calcium-stimulated ATPase in the sarcolemmal membrane of dog (2) and rat (3) myocardium suggests a transport system for calcium similar to what has been described for the erythrocyte membrane (4-7). Such a system would be consistent with the current hypotheses that, in addition to calcium shifts among intracellular pools, active transport across the plasma membrane may regulate the cytoplasmic Ca^{++} levels in the heart (8-11). Based on the observation that epinephrine stimulates Ca^{++} influx (12,13), several authors have proposed that such transport may be controlled by cyclic AMP (8,14). Similarly, Robison et al. (15) favoured such a hypothesis in order to explain the dissociation between the activation of phosphorylase kinase and phosphorylase in rat heart under epinephrine (16). Recent work from this laboratory with a Ca^{++}-stimulated ATPase (3) provided an opportunity to test these

hypotheses, and as shown in the present communication, to obtain evidence for such a mechanism in rat heart sarcolemma.

MATERIAL AND METHODS

Isolation of subcellular organelles: Rat heart sarcolemma was prepared according to a recently published method (17,3). Male Sprague-Dawley rats (150-200 g Fa. Wiga, Munich-Ottobrunn) fed ad libitum with a standardized chow diet (Labortierfutter 57 Z, J.Zahn, II, Hockenheim, Germany) were killed by a blow on the head. The beating hearts were quickly excised and immersed in an ice-cold Tris-sucrose buffer (0.25 M sucrose, 0.5 mM EGTA and 20 mM Tris-Cl, pH 7.4). Ventricles were freed of connective tissue and after passing through a Harvard press, were homogenized in 4 vol of Tris-sucrose buffer with a teflon-glass homogenizer for 1 min at low speed. The homogenate was sedimented at 2300 g for 15 min in a Sorvall super-speed RD 2/B refrigerated centrifuge at 3°C. The pellet was resuspended in the same volume of buffer and washed six times with decreasing time and g-forces down to 470 g x 3 min. After the second wash the homogenate was filtered through cheese-cloth. The ensuing preparation of fibers, corresponding to 1 g of fresh ventricular muscle was suspended in 5 ml of 0.9% NaCl and then 5 ml of a solution containing 6 ml of 6.6 M NaJ, 50 mM cysteine, 5 mM $MgCl_2$, 3 mM ATP and 5 mM EDTA (pH adjusted to 7.4 with Tris-Cl) were slowly added under mechanical stirring at 0°C. After 60 min the mixture was brought to a NaJ-concentration of 0.8 M with distilled water and centrifuged for 20 min at 4300 g. The pellet was then washed twice with 5 mM EDTA-Tris buffer, pH 7.4. The resulting sediment was taken up in distilled water and could be stored for several weeks at -20°C with only small losses of activity. Mitochondria were collected by centrifuging the 2300 g supernatant at 15 900 g for 12 min in the same refrigerated centrifuge. After adjusting the 15 900 supernatant to 0.6 M with KCl, it was spun for 60 min at 220 000g in a type 60 Ti rotor of a Spinco L 2, 65 B ultra-centrifuge in order to obtain a microsomal fraction. Both fractions were washed at the respective g-forces. Suspension of fragmented organelle fractions were prepared in distilled water by homogenisation with a glass-teflon homogenizer containing approx. 5 mg protein/ml.

Analytical procedures: Nucleotides were dissolved in 20 mM, triethanolamine-HCl-buffer maintaining a pH of 7.4, the concentrations ranging from 10^{-7} - 10^{-3}M. Either 0.01 ml of the nucleotide solution or buffer alone were added to 0.02 ml aliquots of the organelle preparations and incubated for 10 min at 37°C in 1.5 ml polyethylene cups. From these 0.02 ml were transferred and assayed for ATPase activity in a reaction mixture of 3 mM ATP (Tris-salt, pH 7.4), 5 mM Tris-Cl, pH 7.4, 0.5 mM EDTA-Tris pH 7.4 and $CaCl_2$, $MgCl_2$ or NaCl and KCl, as indicated. The total volume was 0.1 ml. There was no appreciable increase in Ca^{++}-stimulated ATPase activity without added EDTA. The reaction was linear over 60 min at 37° and was then stopped by adding 0.02 ml of 3.3 M TCA. Phosphate release was measured according to Fiske and SubbaRow (18).

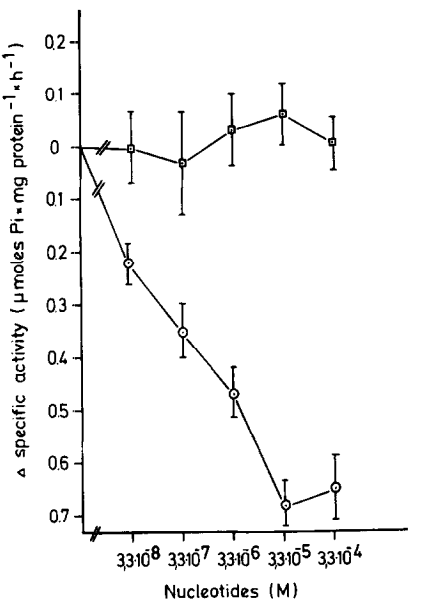

Fig. 1: Dose-response relationship of cyclic AMP and 5'-AMP on calcium stimulated ATP hydrolysis in rat heart sarcolemma.

All values represent the change of specific activity in μmoles Pi released per mg protein per hour as the mean ± S.E.M. of 6 different enzyme preparations in presence of 3'5'AMP o——o and of 4 different preparations in presence of 5'-AMP □——□. The maximal effect could be observed at 3.3 x 10^{-5}M 3'5'-AMP, representing 33.8% inhibition from a control of 2.01 ± 0.15 μmoles Pi x mg protein^{-1} x hour^{-1}. The nucleotide concentrations refer to concentrations in the preincubation medium.

Protein was determined by a biuret method using Labtrol (DADE, Miami, Florida) as a standard (19). Succinate dehydrogenase (E.C. 1.3.9.9.1.) activity was measured according to a modification of the method of Singer (20) and glucose-6-phosphatase (E.C. 3.1.3.9.) according to Baginsky (21).

Chemicals: Adenosine triphosphoric acid was obtained from the Th.Schuchardt Co. Munich. 3'5'adenosine monophosphoric acid and 5'adenosine monophosphoric acid were products of Boehringer-Mannheim. All other reagents were from E.Merck, Darmstadt.

RESULTS

Fig I shows the dose-dependent inhibition of Ca^{++}-stimulated ATP-hydrolysis by 3'5'-AMP over a concentration range from 3.3×10^{-8} to 3.3×10^{-5}M after incubation of the sarcolemmal preparation with the nucleotide for 10 min prior to the ATPase assay. The effect was reproduced with six different membrane preparations involving at least two hearts. While as little as 3.3×10^{-8}M 3'5'-AMP produced a significant effect ($p < 0.005$, paired comparison), maximal inhibition by 33.8% was reached with a concentration of 3.3×10^{-5}M, which represented a mean difference from control of 0.68 µmoles Pi per hour per mg protein, the absolute specific activities being shown in table I. In contrast, previous incubation with equal concentrations of 5'-AMP brought only small and insignificant deviations from the control value (fig. I and table I). When the effect of the nucleotide was tested upon Mg^{+} and Na^{+}, K^{+}-stimulated ATPase activity in the same preparations, no change was observed (table II). The characteristics of a Mg^{+} and Na^{+},K^{+}-ATPase in the present preparation have been bescribed in an earlier report from this laboratory (3); they are similar to what is known of such enzyme activities in sarcolemmal preparations (17). ^{32}Pi incorporation studies (22) with this ATPase suggested phosphorylation and dephosphorylation in analogy to what is known for other Na^{+}, K^{+} ATPases (23).

In view of the stimulation of ATP hydrolysis by Ca^{++} in other subcellular fractions from rat myocardium (24,25,26) it seemed of interest to test the effect of cyclic AMP with a mitochondrial and a microsomal preparation. Indeed, as shown

Table I. Effect of 5'-AMP and 3'5'-AMP on calcium activated ATPase of cardiac sarcolemma

	Nucleotide Concentration (M)					
	Control	3.3×10^{-8}	3.3×10^{-7}	3.3×10^{-6}	3.3×10^{-5}	3.3×10^{-4}
5'-AMP	1.826 ± 0.058	1.863 ± 0.142	1.813 ± 0.043	1.893 ± 0.066	1.910 ± 0.115	1.850 ± 0.085
3'5'-AMP	2.012 ± 0.155	1.789 ± 0.155	1.682 ± 0.168	1.541 ± 0.138	1.332 ± 0.138	1.404 ± 0.141

All values represent the specific activity in micromoles Pi x mg protein^{-1} x hour^{-1}, the 5'-AMP series as the mean of 4 preparations ± S.E.M., the 3'5'-AMP series as the mean of 6 preparations ± S.E.M. $CaCl_2$ and ATP concentrations were 0.6 and 3 mM, respectively. Basal values (no Ca^{++} added) have been subtracted. The Ca^{++} concentration was selected to cause submaximal stimulation of ATPase activity (3). The mean basal value without added Ca^{++} was 1.667 ± 0.073 micromoles Pi x mg protein^{-1} x hour^{-1}; nucleotide concentrations refer to the preincubation period.

Table II Effect of 3'5'-AMP on Mg^{++}-, Na^{+},K^{+}-ATPase of rat heart sarcolemma

	Control	3'5'-AMP 10^{-6}M
5 mM $MgCl_2$	1.995 ± 0.301	1.965 ± 0.348
5 mM $MgCl_2$ 140 mM NaCl 14 mM KCl	5.91 ± 0.395	5.99 ± 0.539

All values represent the specific activity in micromoles Pi x mg protein^{-1} x hour^{-1} as the mean of 3 different preparations ± S.E.M. 3'5'-AMP concentration is indicated for the preincubation period. Assay of respective ATPase activity as described previously (3).

Table III. Influence of cyclic AMP on Ca^{++}-ATPase of different myocardial cell organelles

	Sarcolemma	Mitochondria	Microsomes
0.6 mM $CaCl_2$	2.012 ± 0.156	3.875 ± 0.338	6.570 ± 0.282
0.6 mM $CaCl_2$ + 3.3×10^{-5}M;3'5'-AMP	1.541 ± 0.148[a)]	2.172 ± 0.471[a)]	9.910 ± 0.583[a)]

All values represent the specific activity in micromoles Pi x mg $protein^{-1}$ x $hour^{-1}$ ± S.E.M. of calcium activated hydrolysis of sarcolemma as the mean of 6 preparations, of mitochondria as the mean of 5 preparations and of microsomes as the mean of 3 preparations. [a)Difference versus control p< P-0.005, paired t-test] 0.6 mM $CaCl_2$ represents a submaximal stimulation of sarcolemma and halfmaximal of mitochondrial and microsomal Ca^{++}-ATPases. 3'5'-AMP was preincubated with each fraction as described under methods.

in table III, the cyclic nucleotide was found to exert significant effects, inhibiting ATPase activity in the mitochondrial and stimulating it in the microsomal fraction. As can be derived from a previous communication (3), ATPase activity in all three subcellular fractions showed clearly different characteristics.

DISCUSSION

The present report describes an inhibitory effect of cyclic AMP on a recently described Ca^{++}-activated ATPase which is likely to be involved in active transport of calcium across the sarcolemmal membrane of the heart (8-11). Thus far the data provide evidence for an individual Ca^{++}-activated enzyme which appears to be different from ATPase activities in mitochondrial, microsomal and myosin filament fractions of the myocardium (3). In view of the dose-dependency over a range of nucleotide concentrations which would correspond to actual levels of cyclic AMP in rat heart as measured by several different methods under basal conditions as well as catecholamine stimulation (27,28) and in view of the similarity to dose-response curves that have been described for various 3'5'-AMP-dependent protein kinases (29,30), it seems justified to discuss the physiological significance of the present observation. The fact that Mg^{++} and Mg^{++}, Na^{+}-K^{+}-ATPase activities remained unchanged, would argue against the possible diversion of substrate from the ATPase to an indepen dent protein kinase also present in the same fraction. However, it seems not unlikely that 3'5'-AMP dependent phosphokinase is in fact a subunit of the Ca^{++}-ATPase system under study, and that a dephosphorylation step in analogy to what has been shown to occur under the influence of K^{+} in the Na^{+}, K^{+}-ATPase system (22,23), may be counteracted by phosphorylation in the presence of cyclic AMP. It is not clear why the maximal inhibition on the average was not greater than 34%. This could be due to microsomal contamination which would introduce an ATPase activity that can be stimulated by cyclic AMP (table III) thus counteracting the inhibitory effect. That treatment with NaJ has led to a diminished sensitivity toward cyclic AMP seems unlikely since it was observed that the duration of NaJ treatment had no effect upon the extent of the inhibition.

Although other workers have failed to demonstrate a significant effect of cyclic AMP on Ca^{++}-ATPase activities of microsomal preparations (31), the stimulation described herein is not surprising in view of the well-documented increase in Ca^{++} uptake of microsomes in presence of the cyclic nucleotide (31). These results with the microsomal and the mitochondrial fractions, though only preliminary, would suggest regulation of intracellular calcium movement by other cyclic AMP sensitive ATPase systems.

There is little doubt that calcium in connection with cyclic AMP plays an important role as transmitter in different cellular functions (14). For instance, it seems well established that calcium is instrumental in excitation-contraction coupling (9-11). While it was first believed that the cytoplasmic calcium level is mainly regulated by shifts between intracellular calcium pools (24-26), more observations favour additional control by transmembrane calcium transport (1,3,32). The studies of Nayler (12) and of Fleckenstein (13) suggest hormone sensitivity of transmembrane calcium flux. The present data, in support of these results, provide further insight into the mechanism of the propranolol- sensitive increase of intracellular calcium under the influence of epinephrine (12), and suggest that cyclic AMP links the hormone-sensitive adenyl cyclase system to active calcium transport in the sarcolemma.

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