LOCALIZATION OF THE CYCLIC ADENOSINE 3' : 5' - MONOPHOSPHATE PHOSPHODIESTERASE ACTIVATOR PROTEIN IN RAT HEART

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SUMMARY: A cyclic adenosine 3' : 5' - monophosphate phosphodiesterase activator protein has been partially purified from rat heart by a procedure involving ammonium sulfate fractionation and affinity column chromatography with cyclic AMP phosphodiesterase bound to Sepharose 4B. Freezing and thawing of the rat heart was essential for solubilization of the activator protein in the crude homogenate. Activator activity was localized on sarcoplasmic reticulum isolated from fresh heart which could be solubilized with a low yield that resulted in a labile product. Maximal activation of cyclic AMP phosphodiesterase with excess protein activator was 100%.

The function of cyclic AMP as a mediator ("second messenger") of hormonal action is now firmly established (1). In the case of cardiac muscle, the "second messenger" may, in turn, regulate calcium metabolism or the calcium metabolism may be affected by the "first messenger" (catecholamines) (2). Whatever the exact mechanism of cardiac control, the hormonal cardiac response changes the beating rate, the force of contraction and the glycogenolysis.

In order to develop a basic understanding of the control of physiological and biochemical reactions by cyclic AMP, the regulation of cyclic AMP degradation must be elucidated. Cyclic AMP phosphodiesterase was first demonstrated in 1958 (3). However, only recently has the existence of a protein modifier been reported for cyclic AMP phosphodiesterase which requires Ca^{2+} as a cofactor (4,5,6).

The involvement of Ca^{2+} in the regulation of cyclic AMP levels was indicated in intact tissue by Namm et al. (7) who observed that the cyclic AMP concentration in perfused rat hearts increased more with Ca^{2+} - free medium than with Ca^{2+} - rich medium when stimulated with norepinephrine. Further investigation in the delineation of calcium control of cyclic AMP led us to study the calcium requirement for cyclic AMP phosphodiesterase.

The intent of this communication is to report the possible localization of rat heart cyclic AMP phosphodiesterase and some of its properties.

MATERIALS AND METHODS

5' - Nucleotidase, grade IV, partially purified from Crotalus atrox venom, Sepharose 4B, and adenosine deaminase were purchased from Sigma, DEAE-cellulose (DE52) from H. Reeve Angel, and cyclic AMP from P and L.

Activator-deficient cyclic AMP phosphodiesterase was prepared by the

following method: Hearts from decapitated adult rats (Wistar HLA-W) were excised and immediately cooled to 0°C. All following procedures were performed between 0° and 4°C. Dissected ventricles were washed with 0.1 M Tris-HCl + 2 mM EDTA (pH 7.5). This tissue was diced and homogenized in four volumes of the same buffer with a Virtis homogenizer at a moderate speed. The crude extract was centrifuged at 48,200 x g for 25 min. The supernatant was made 35% saturating in ammonium sulfate. The pellet resulting from centrifugation for 20 min at 10,000 x g was dissolved in a minimal volume of 0.02 M Tris-HCl, 0.08 M NaCl, 1 mM $MgCl_2$ and 1 mM imidazole (pH 7.5) buffer. After extensive dialysis with the same buffer the preparation was centrifuged at 100,000 x g for 1 hr. The supernatant was applied to DEAE-cellulose column (1.5 x 21 cm) and eluted with a linear salt gradient from 0.08 to 0.65 M NaCl in 0.02 M Tris-HCl, 1 mM $MgCl_2$ and 1 mM imidazole (pH 7.5) (Fig. 1). Fractions were pooled and concentrated with a Millipore Ultrafiltration Cell utilizing a type PSED membrane (25,000 nominal molecular weight limit). The concentrated protein solution was dialyzed against 0.02 M Tris-HCl, 1 mM $MgCl_2$ and 1 mM imidazole (pH 7.5). (See Table I.)

Activator-deficient cyclic AMP phosphodiesterase was bound to Sepharose 4B by the following method: Sepharose 4B (25 ml) was washed with 50 ml of 1 M NaCl followed with 100 ml of cold glass distilled water. The gel was added to 25 ml of cold glass distilled water containing dissolved cyanogen bromide (0.75 g). The pH was immediately raised to 11 with 3 M NaOH and maintained at 11 with further additions of NaOH for 9 minutes. The mixture was added to 300 ml of glass distilled water at 0°C, quickly filtered on a Buchner funnel

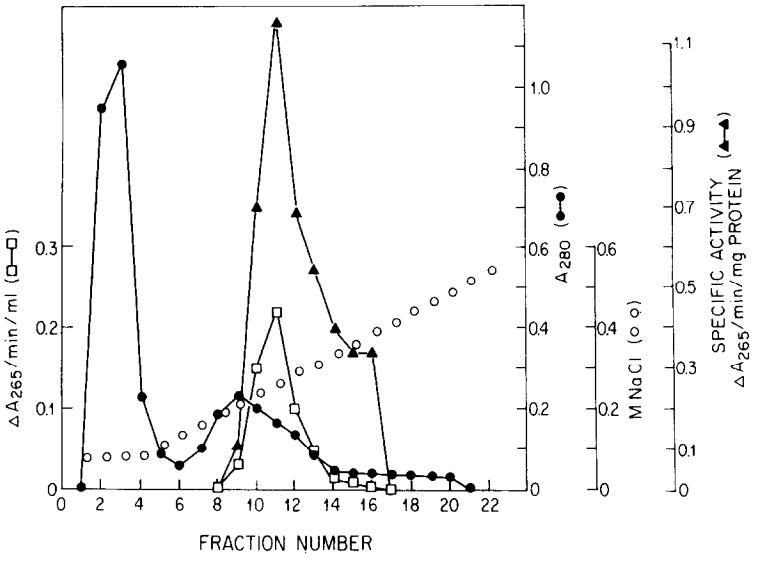

FIG. 1. Results of adsorption chromatography for activator-deficient cyclic AMP phosphodiesterase. To a DEAE-cellulose column (1.5 x 21 cm), 23 mg of protein from the 100,000 x g step were added. The protein was washed into the column with 10 ml of 0.02 M Tris-HCl, 0.08 M NaCl, 1 mM $MgCl_2$ and 1 mM imidazole (pH 7.5) buffer. The column was eluted with a linear gradient generated from 100 ml of 0.08 M NaCl and 100 ml of 0.65 M NaCl in 0.02 M Tris-HCl, 1 mM $MgCl_2$ and 1 mM imidazole. Fractions of 8.5 ml were collected. The flow rate was 15 ml/hr. Enzyme activity was measured as described in the text. The closed circles indicate A_{280} of the elluent. The triangles represent specific activity, and the open circles indicate the molarity of the NaCl gradient.

TABLE I

Partial purification of activator-deficient cyclic AMP phosphodiesterase

Step	Activity[b] ΔA_{265}/min/ml	Total Activity	Protein mg/ml	Specific Activity	Yield %	Purification
Crude extract[a]	0.40	11.60	12.5	0.032	100	1.0
35% pellet	1.62	5.67	14.9	0.108	49	3.4
Dialysis 100,000 x g	1.05	3.20	7.47	0.140	28	4.4
Pooled fractions 10-13 from DEAE cellulose column and concentrated	0.86	3.04	1.07	0.800	26	25.0

a 9.11 g of fresh ventricles were homogenized
b. Assayed as described in text

with suction, washed with 400 ml of 0.1 M $NaHCO_3$ (pH 8.5), and added to 25 ml of 0.1 M $NaHCO_3$ (pH 8.5) which contained 3 mg of partially purified activator-deficient phosphodiesterase. This mixture was stirred overnight (0-4°C). The mixture was then filtered on a Buchner funnel with suction and washed with 0.02 M Tris-HCl, 0.7 M NaCl, 1 mM $MgCl_2$ and 1 mM imidazole (pH 7.5).

Various membranes were prepared by the following method: The crude extract was prepared as in the preparation of the activator-deficient phosphodiesterase. It was centrifuged for 10 min at 270 x g. The resulting supernatant was centrifuged at 48,200 x g for 25 min. This pellet was suspended in a minimal volume of 0.25 M sucrose, and layered on top a linear sucrose gradient varying from 30% to 45% sucrose. Plasma membranes, sarcoplasmic reticulum and mitochondrial fractions were separated according to the method of Kidwai et al. (8) by centrifuging the crude membranes for 90 minutes at 96,300 x g. The top band at the interphase of the 0.25 M sucrose and the sucrose gradient is composed of plasma membranes. The second band was mainly sarcoplasmic reticulum while the thick sharp band in the middle of the tube was mitochondria.

Cyclic AMP phosphodiesterase was assayed spectrophotometrically by converting AMP to inosine by the combined activities of 5'-nucleotidase and adenosine deaminase. The absorption spectra of the reaction substrate and intermediates are indistinguishable while the deamination of adenosine is accompanied by a decrease in absorbance at 265 nm (extinction coefficient of 8060 $M^{-1} cm^{-1}$ (9)). The assay was performed at 30°C and the assay mixture consisted of 60 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 0.1 mM cyclic AMP, 0.5 units 5' - nucleotidase and 0.5 units adenosine deaminase. The assay mixture contained 7 μM Ca^{2+} as measured by atomic absorbtion and this was enough for full activation. Its source was from the 5' - nucleotidase. Protein was measured by the method of Warburg and Christian (10).

RESULTS

The stimulation of phosphodiesterase activity by Ca^{2+} is dependent on the presence of activator. In its absence the base line activity of phosphodiesterase is not affected by Ca^{2+}. Activator is assayed by its addition to phosphodiesterase and by measurement of the increment caused by Ca^{2+}.

Endogenous activator in the presence of phosphodiesterase can be assayed by

TABLE II

Comparison of decrease in cyclic AMP phosphodiesterase activity in fresh and frozen-thawed preparations

Assay	Activity[a] ΔA_{265}/min/mg protein
Fresh preparation	0.026
Fresh preparation + 0.5 mM EDTA	0.026
Fresh preparation one day old	0.027
Fresh preparation one day old + 0.5 mM EDTA	0.027
Frozen and thawed preparation	0.038
Frozen preparation + 0.05 mM EDTA	0.030
Frozen preparation one day old	0.034
Frozen preparation one day old + 0.5 mM EDTA	0.027
Frozen preparation two days old	0.028
Frozen preparation two days old + 0.5 mM EDTA	0.028

[a]Cyclic AMP Phosphodiesterase activity was measured as described in the text.

the addition of EDTA. The increment caused by Ca^{2+} is decreased by EDTA and is a measure of the activator. The value in the presence of EDTA decreased to the activity of the unstimulated phosphodiesterase.

Fresh preparations of rat heart ventricle contained phosphodiesterase but never had any measurable activator. EDTA had no affect on the phosphodiesterase activity (Table II). Freezing and thawing of the ventricle was a prerequisite for the preparation of the extract which contained both phosphodiesterase and activator. Phosphodiesterase activity in preparation of frozen and thawed ventricles decreased over a period of two days to a stable value. This loss of activity reflected the loss of activator since in the presence of EDTA, the phosphodiesterase activity was reduced to the low stable value throughout the two-day period. The base line nonactivated phosphodiesterase activity appeared to be stable in both fresh and frozen and thawed preparations. The total activity of the phosphodiesterase from frozen tissues decreased after two days to the activity observed with the fresh preparation. The activity remaining after this time was not affected by EDTA. It is thus indicated that freezing of the ventricles solubilized the activator and that the activator is unstabl

Preparation of Cyclic AMP Phosphodiesterase Activator:

Ventricles from adult rat hears were cooled to 0°, washed with 0.1 M Tris-HCl + 2 mM EDTA (pH 7.5), and frozen at -20°C. The following work was done between 0° and 4°C. The ventricles were slowly thawed, diced and homogenized at a moderate speed with a VirTris homogenizer in four volumes of the above buffer. The crude extract was centrifuged at 48,200 x g for 25 min. The super-

TABLE III

Partial purification of cyclic phosphodiesterase activator protein

Step[a]	Activity[b] unit/ml	Total Activity	Protein mg/ml	Specific Activity	Yield %	Purification	Phosphodiesterase Activity ΔA_{265}/min/ml
Crude extract	158	610	12.50	4.7	100	1.0	0.35
35%-60% ammonium sulfate	146	220	24.90	5.9	36	1.2	1.20
Affinity column	11	55	0.12	92.0	9	19.5	--

a 3.50 g frozen ventricles.
b Unit of activity for phosphodiesterase activator is defined with the results. Assay conditions are described in the text.

natant was made 35% saturating in ammonium sulfate and centrifuged for 20 min. at 10,000 x g. The resulting supernatant was removed and made 60% saturating in ammonium sulfate and centrifuged for 20 min. at 10,000 x g. The pellet was dissolved in a minimal volume of 0.03 M imidazole + 1 mM Mg acetate (pH 6.5) and dialyzed against the same buffer for two hours. The dialyzed protein was added to a 0.7 x 25 cm column containing activator deficient phosphodiesterase bound to Sepharose 4B previously equilibrated with 0.03 M imidazole + 1 mM Mg acetate (pH 6.5). The column was washed with 8 ml of the same buffer and eluted with 5 ml of 0.17 M NaCl and 10 ml of 0.7 M NaCl both of which contained 0.03 M imidazole +1mM Mg acetate (pH 6.5). The phosphodiesterase activator was located in the 0.7 M NaCl fraction which was concentrated with a Millipore Ultrafiltration cell utilizing a type PSAC membrane (1,000 nominal molecular weight limit). The concentrated phosphodiesterase activator was dialyzed against 0.03 M imidazole + 1 mM Mg acetate (pH 6.5) (See Table III).

The cyclic AMP phosphodiesterase reaction was doubled at the saturating concentration of activator (Fig. 2). One unit of activator activity is defined as the amount of activator required for half-maximal activation at a standard concentration of cyclic AMP phosphodiesterase and of cyclic AMP.

Localization of Cyclic AMP Phosphodiesterase Activator

The necessity for freezing and thawing the ventricles in order to solubilize the activator protein suggested that the activator may be associated with a membrane in the intact cell. Therefore, membrane fractions were prepared from fresh heart to determine whether the activator protein was associated with any of the cellular membranes. Membrane fractions were prepared as previously indicated.

The assay for the particulate activator was performed as with the soluble activator. Small quantities of membrane were used since larger amounts caused inhibition. The apparent activity of mitochondrial membranes was corrected for a time dependent change in light scattering caused when the membranes were

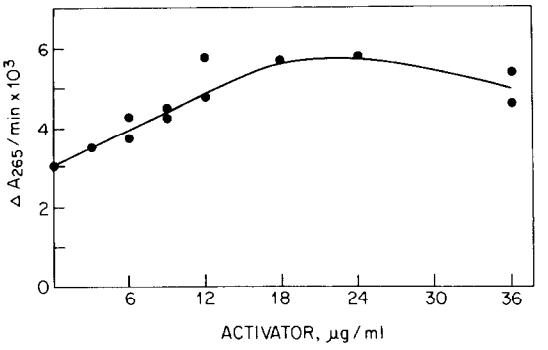

FIG. 2. Activation of activator-deficient cyclic AMP phosphodiesterase by activator protein. The assay conditions are described in the text. Cyclic AMP phosphodiesterase concentration was 5.4 μg/ml with a specific activity of 0.55 ΔA_{265}/min/mg protein.

added to the reaction mixture. This correction factor was measured in the absence of cyclic AMP phosphodiesterase. Plasma membranes and mitochondrial membranes showed no activity. Activator activity was found in the sarcoplasmic membranes but only if fresh ventricles were used in the membrane preparations; no particulate activator was found in a frozen-thawed ventricle preparation. Fig. 3 shows an electron micrograph of sarcoplasmic membranes. There is a slight mitochondrial contamination but this is not the source of the activator activity since the mitochondria fraction had no activity. The activator was solublized from the sarcoplasmic membrane by three different methods; (1) The sarcoplsmic membrane was frozen at -20°C in equidensity sucrose; the sample thawed, diluted with 0.02 M Tris-HCl, 0.08 M NaCl, 1 mM $MgCl_2$ + 1 mM imidazole (pH 7.5) buffer and centrifuged at 48,000 x g for 20 min. (2) The sarcoplasmic membrane was heated at 65°C for 45 s, rapidly cooled with ice and centrifuged. (3) After four-fold dilution of the membrane suspension with distilled water followed by 40,000 x g centrifugation for 20 min., the pellet was resuspended in 0.1 M Tris-HCl + 1 mM EDTA (pH 7.5) containing 35% saturation ammonium sulfate (followed by centrifugation). The solubilized activator prepared by any one of these three methods was unstable and was completely lost within 24 hours compared to 48 hours for the frozen ventricular preparation.

Discussion

As reported here, rat heart contains a phosphodiesterase activator as does beef heart (5). The amount of phosphodiesterase activation elicited by the activator was 100% increase for the rat heart. This is about the same value reported by one group for the beef heart (11) but different from the 6 to

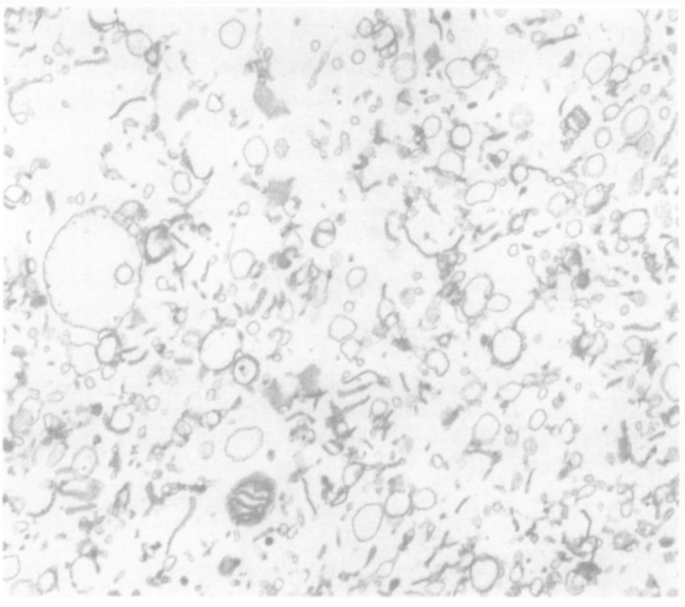

FIG. 3. Electron micrograph of sarcoplasmic reticulum magnified 18,000 times. The membranes were fixed as a very thin pellet in 2.5% glutaraldehyde in 0.1 M Na_2HPO_4 (pH 7.2) buffer and post osmicated in 1% OsO_4. They were embedded in Luft's epon, and sections were taken across the entire thickness of the pellet. The area shown is representative of the entire pellet.

10-fold activity also reported in beef heart (17). One difference in the two groups is that the former used fresh while the latter used frozen preparations. Freezing and thawing may play a role in the nature and yield of activator. Species differences may also play a role. Beef brain activator gives a 6-fold maximal activation (4) whereas rat brain results in 100% increase in phosphodiesterase (13). Thus, rat brain and heart may have intrinsically lower activator activity than beef brain and heart.

The interrelationship between cyclic AMP and Ca^{2+} has been discussed in a review by Rasmussen et al. (2). It is not known how the relationship of cyclic AMP to Ca^{2+} affects myocardial contraction. Ca^{2+} may regulate the micro-environmental level of cyclic AMP. On the other hand cyclic AMP may control cellular uptake of Ca^{2+}. It has been shown to stimulate Ca^{2+} uptake by canine cardiac microsomes in the presence of cyclic AMP dependent protein kinase (14).

The sarcoplasmic reticulum regulates the Ca^{2+} level for cardiac contraction and cyclic AMP may also play a role in this regulation. If cyclic AMP does this by affecting Ca^{2+} uptake and Ca^{2+} in turn affects the cyclic AMP level it is logical to have the Ca^{2+} activator phosphodiesterase localized in the sarcoplasmic reticular membrane. Changes in Ca^{2+} could affect phosphodiesterase activity in

a location where changes in the cyclic AMP are significantly related to that needed for altered Ca^{2+} uptake. In support of this, Brooker (15) has reported that cyclic AMP varies during the cardiac cycle where Ca^{2+} levels vary to induce contraction and relaxation.

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