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CYCLIC 3′:5′-NUCLEOTIDE PHOSPHODIESTERASE OF RABBIT SINOATRIAL NODE

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Summary

The sinoatrial node contains pacemaker cells which control heart rate. Changes of heart rate correlate closely to cyclic adenosine 3′:5′-monophosphate (cyclic AMP) levels in sinoatrial node. In order to elucidate the mechanism of regulation of cyclic AMP level in sinoatrial node, we investigated cyclic 3′:5′-nucleotide phosphodiesterase (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) of isolated rabbit sinoatrial node.

Sinoatrial node of rabbit heart contains at least three kinetically distinct forms of cyclic nucleotide phosphodiesterase (F I, F II and F III) which were clearly separated by DEAE-cellulose column chromatography. Although phosphodiesterase F I and F II hydrolyzed cyclic GMP faster than cyclic AMP at a low substrate concentration, phosphodiesterase F III hydrolyzed equally cyclic GMP and cyclic AMP at a low substrate concentration. Phosphodiesterase F I displayed normal kinetics both for cyclic AMP hydrolysis, with a K_m of 8.3 μM, and for cyclic GMP hydrolysis, with a K_m of 2.0 μM. Phosphodiesterase F II and F III displayed abnormal kinetics both for cyclic AMP, with a K_m for 1.2 and 0.8 μM, respectively, and for cyclic GMP, with a K_m of 2.0 and 0.1 μM, respectively.

The effects of the activator, Ca^{2+}, cyclic AMP and cyclic GMP on these forms were also studied. The cyclic GMP and cyclic AMP hydrolytic activities in phosphodiesterase F I and F II were stimulated by micromolar concentration of Ca^{2+} in the presence of the activator. Phosphodiesterase F III was not affected by addition of Ca^{2+} in the presence of the activator. Cyclic AMP hydrolysis by phosphodiesterase F I and F II was activated by micromolar amounts of cyclic GMP, whereas cyclic GMP hydrolysis by these fractions was inhibited by micromolar amounts of cyclic AMP. Cyclic AMP hydrolytic activity in phosphodiesterase F III was inhibited by micromolar amounts of cyclic GMP, and cyclic GMP hydrolysis by phosphodiesterase F III was also inhibited by cyclic AMP.

These results suggest that the activity of cyclic nucleotide phosphodiesterase in sinoatrial node was complicatedly controlled by the activator, and micro-amounts of Ca^{2+}, cyclic GMP and cyclic AMP.

Atrial and ventricular cyclic nucleotide phosphodiesterase were briefly studied and their properties were compared to those of sinoatrial node enzyme.

Introduction

Cyclic AMP and cyclic GMP appear to play a most important role in many tissues and affect the intracellular levels of these nucleotides which have profound effects on the function of a tissue. These levels are controlled by both adenylate cyclase (or guanylate cyclase) and cyclic nucleotide phosphodiesterase (3′:5′-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17). In our previous report [1], it was suggested that the cardiac positive chronotropic effect of catecholamine is the result of an increase in content of cellular cyclic AMP in sinoatrial nodal pacemaker cells. Recently, Goshima [2] showed that noradrenaline and dibutyryl cyclic AMP accelerated the rate of spontaneous beating in cultured myocardial cells obtained from neonatal mouse ventricles, and the acceleration of the beating rate of cultured myocardial cells by noradrenaline or dibutyryl cyclic AMP was counteracted by dibutyryl cyclic GMP. Thus, cyclic AMP and cyclic GMP metabolism plays an important role in chronotropism of the heart.

The present study was undertaken to study the properties of cyclic nucleotide phosphodiesterase of rabbit sinoatrial node.

Materials and Methods

Materials. Cyclic [^{3}H]adenosine 3′:5′-monophosphate (specific activity: 30 Ci/mmol) and cyclic [^{3}H]guanosine 3′:5′-monophosphate (8.44 Ci/mmol) were purchased from New England Nuclear. Unlabeled cyclic AMP, cyclic GMP and snake venom (*Crotalus atrox*) were purchased from Sigma Chemical Co. Cation-exchange resin (BioRad AG 50W-X4, 200—400 mesh) was extensively washed with 0.5 M NaOH, 0.5 M HCl and deionized water to final pH of 5.0. DEAE-cellulose (Whatman, DE-52) was equilibrated with the column buffer prior to use. All other chemicals were reagent grade or the best commercially available.

Isolation of sinoatrial node and preparation of soluble phosphodiesterase. Sinoatrial node, atria and ventricle of rabbit heart were isolated according to the method of Taniguchi et al. [1]. Albino rabbits of both sexes, weighing 1.8—2.3 kg, were stunned by a blow on the neck and killed by exsanguination from the common carotid arteries. The heart was removed and the sinoatrial nodal, atrial and ventricular preparations were dissected free. The contents of noradrenaline and cyclic AMP, and the density of fluorescent adrenergic nerve terminals in the sinoatrial node preparation were higher than in the rest of the atrium [1]. The tissues were stored —20°C until homogenization. The homogenates were prepared in 50 mM Tris · HCl buffer (pH 7.4) containing 1 mM $MgCl_2$ using a glass homogenizer. Soluble cyclic nucleotide phosphodiesterase preparation was obtained by centrifugation at 105 000 × *g* for 60 min and this

preparation was used for chromatographic studies.

Phosphodiesterase assay. The two-step assay for enzymatic activity is similar to that previously described [3]: 5′-[^{3}H]AMP or 5′-[^{3}H]GMP formed by the phosphodiesterase is converted to [^{3}H]adenosine or [^{3}H]guanosine by the action of nucleotidase and the product which is isolated by cation-exchange resin is counted in a liquid scintillation counter. An appropriate dilution of enzyme is incubated in 50 mM Tris · HCl (pH 8.0)/50 mM $MgCl_2$/50 μg of bovine serum albumin/0.4 μM cyclic [^{3}H]AMP or 0.4 μM cyclic [^{3}H]GMP, in total volume of 0.5 ml. When higher concentrations of substrate are required, indicated amounts of unlabeled cyclic nucleotide are incubated. After 20 min at 30°C, the reaction mixture is terminated by boiling for 5 min. Next 0.05 ml of snake venom (1 mg/ml in H_2O) is added with an additional incubation for 10 min at 30°C. Then 1.0 ml of water is added and the mixture applied to a small ion-exchange resin column (AG 50W-X4, 0.8 × 2 cm column). Adenosine or guanosine is eluted with 1.5 ml of 3 M NH_4OH after washing the column with 15 ml of distilled water. Recovery of adenosine or guanosine from the column is 95%. When a crude preparation (105 000 × *g* supernatant) was used as the enzyme source above 80% of the isolated radioactive product formed enzymatically from cyclic [^{3}H]AMP was confirmed to be [^{3}H]adenosine by thin-layer chromatography [3] while 100% of the radioactivity in this assay was found to be due to [^{3}H]adenosine when the preparation from DEAE-cellulose column was used as an enzyme. On the other hand, when cyclic GMP was used as the substrate, [^{3}H]guanosine was the only breakdown product when both crude and DEAE-cellulose preparations were used. Accordingly, the estimation of cyclic AMP hydrolysis by 105 000 × *g* supernatant was performed by using anion-exchange column by the method of Rutten et al. [4].

Chromatography. DEAE-cellulose chromatography was performed by the method of Russell et al. [5] in columns (1.5 × 2.0 cm) with bed volumes of 35 ml. The buffer was 50 mM Tris/acetate, pH 6.0, containing 3.75 mM 2-mercaptoethanol. The enzyme preparation was applied to the column, followed by elution with several column volumes of buffer. The initial wash contained no detectable phosphodiesterase activity. A linear gradient from 0 to 1 M sodium acetate was then applied with a flow rate of 0.05 ml per min and a total gradient volume of 300 ml. Fractions were pooled and concentrated by ultrafiltration using collodion bags. The concentration by ultrafiltration was performed only when it was necessary, because a large loss (approx. 50%) of activity occurred, probably due to inactivation during concentration by ultrafiltration. The enzyme activity was more labile when the preparation was diluted. Purified enzyme preparations were usually stored at 4°C in the presence of 0.1 mg/ml of bovine serum albumin and, in this condition, 50% of the activity of purified preparation (phosphodiesterase F I, FII and FIII) remained after 3 weeks.

Preparation and assay of the activator. The activator of the rabbit brain was prepared according to the method of Cheung [6]. Fresh rabbit brain was homogenized with three volumes of glass-distilled water chilled to 0°C. The sediment was discarded and the supernatant was heated for 5 min in a boiling bath. Denatured proteins were removed by centrifugation and the boiled supernatant was used for the experiments. Assay of the activator was carried out according to the method of Kakiuchi et al. [7] based on the ability of the activator to en-

hance the activity of diluted 105 000 × *g* supernatant fluid of the rat brain homogenate.

Protein determination. Protein was determined by the method of Lowry et al. [8] with bovine serum albumin as a standard.

Results

Distribution of cyclic AMP and cyclic GMP hydrolytic activities in several preparations of the rabbit heart

The 105 000 × *g* supernatant fraction of sinoatrial node was found to contain 28% of the homogenate cyclic AMP hydrolytic activity at a low substrate concentration and 30% at a high substrate concentration, and contained 50% of the homogenate cyclic GMP hydrolytic activity at a low substrate concentration and 27% at a high substrate concentration, respectively. In contrast to sinoatrial node, both cyclic AMP and cyclic GMP hydrolytic activities in the 105 000 × *g* supernatant fraction of atrial and ventricular homogenate were about equal or slightly higher than the activities in the 105 000 × *g* precipitate fractions. These data are summarized in Table I.

The activity for cyclic GMP hydrolysis in the sinoatrial nodal homogenate was 3.8-fold higher than that for cyclic AMP hydrolysis at a low substrate concentration, and cyclic GMP hydrolytic activity in the 105 000 × *g* supernatant was 6.7-fold higher than the cyclic AMP hydrolytic activity at the same substrate concentration, suggesting that the sinoatrial node contains an extremely high cyclic GMP hydrolytic activity in soluble form, as compared to the cyclic AMP hydrolytic activity.

Purification and properties of cyclic nucleotide phosphodiesterase in rabbit sinoatrial node

DEAE-cellulose chromatography of rabbit sinoatrial nodal extract reveals at least three cyclic nucleotide phosphodiesterase fractions designated as phosphodiesterase F I, F II and F III in Fig. 1. Phosphodiesterase F I and F II hydrolyzed cyclic GMP faster than cyclic AMP at both 0.4 and 100 μM substrate concentrations, while phosphodiesterase F III hydrolyzed about equally cyclic GMP and cyclic AMP at both 0.4 and 100 μM substrate concentrations.

Kinetic analysis of cyclic AMP and cyclic GMP hydrolysis by DEAE-cellulose chromatography resolved sinoatrial nodal enzymes was made using Lineweaver-Burk plots. The substrate concentration used to measure the K_m ranged from 0.2 μM to 1 mM. Phosphodiesterase F I displayed normal Michaelis-Menten kinetics both for cyclic AMP hydrolysis and for cyclic GMP hydrolysis. Phosphodiesterase F II and F III displayed abnormal Michaelis-Menten kinetics both for cyclic AMP hydrolysis and for cyclic GMP hydrolysis. The apparent K_m values are summarized in Table II.

Effect of the activator on the cyclic nucleotide phosphodiesterase of sinoatrial node was studied. Addition of the activator to each fraction from DEAE-cellulose chromatography barely altered its elution profile which was obtained by determining the activity without the addition of the activator. The effects are summarized in Table III. Phosphodiesterase F I and F II activities were stimulated by the activator in the presence of Ca^{2+}, but phosphodiesterase F III

TABLE I

DISTRIBUTION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN VARIOUS AREAS OF THE RABBIT HEART

Values indicate the mean ± S.E. of three determinations. Parentheses indicate recoveries of the activity in each fraction.

	Hydrolytic activity (μmol/min/g per tissue)			
	Cyclic AMP		Cyclic GMP	
	0.4 μM	100 μM	0.4 μM	100 μM
Sinoatrial node				
Homogenate [a]	0.032 ± 0.001 [d] (100%)	0.027 ± 0.002 [d] (100%)	0.12 ± 0.01 (100%)	1.47 ± 0.04 (100%)
Supernatant [b]	0.009 ± 0.001 (28%)	0.008 ± 0.001 (30%)	0.06 ± 0.01 (50%)	0.40 ± 0.02 (27%)
Precipitate [c]	0.026 ± 0.002 (81%)	0.018 ± 0.001 (67%)	0.08 ± 0.01 (67%)	1.05 ± 0.03 (71%)
Atria				
Homogenate	0.57 ± 0.02 [d] (100%)	8.33 ± 0.34 [d] (100%)	1.04 ± 0.04 (100%)	10.72 ± 0.36 (100%)
Supernatant	0.29 ± 0.02 (51%)	5.26 ± 0.16 (63%)	0.73 ± 0.09 (70%)	7.88 ± 0.16 (74%)
Precipitate	0.29 ± 0.04 (51%)	3.19 ± 0.74 (38%)	0.32 ± 0.12 (31%)	3.17 ± 0.18 (30%)
Ventricle				
Homogenate	0.55 ± 0.07 [d] (100%)	11.02 ± 0.57 [d] (100%)	1.40 ± 0.04 (100%)	15.95 ± 0.20 (100%)
Supernatant	0.31 ± 0.05 [d] (56%)	5.37 ± 0.57 (49%)	0.66 ± 0.07 (47%)	8.66 ± 0.46 (54%)
Precipitate	0.27 ± 0.01 (49%)	4.88 ± 0.19 (44%)	0.62 ± 0.04 (44%)	6.46 ± 0.39 (41%)

[a] 10% (w/v) homogenate.

[b] 105 000 × *g* supernatant.

[c] 105 000 × *g* precipitate.

[d] Activity were determined by anion-exchange resin according to the method of Rutten et al. [4] (see Materials and Methods).

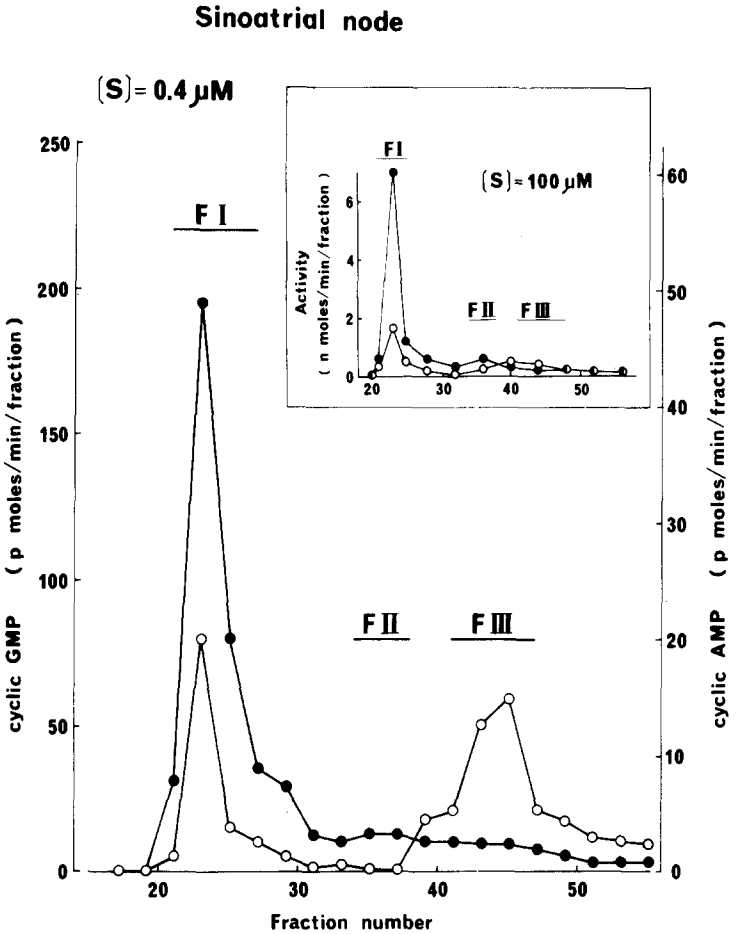

Fig. 1. DEAE-cellulose profile of sinoatrial node phosphodiesterase from 105 000 × *g* supernatant. The gradient was from fraction 20 to 80. Aliquots, 0.2 ml for high substrate (100 μM) or 0.01 ml for low substrate level (0.4 μM), were assayed directly. ○———○, cyclic AMP hydrolysis; ●———●, cyclic GMP hydrolysis.

TABLE II

APPARENT K_m VALUES OF SINOATRIAL NODE, ATRIA AND VENTRICLE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

K_m values were determined by the Lineweaver-Burk plots.

	F I		F II		F III	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Sinoatrial node	8.3 μM	2.0 μM	1.2 μM	2.0 μM	0.8 μM	0.1 μM
			50	20	10	5.9
Atria	1.3	5.0	1.6	2.5	1.2	0.8
	25	50	20	33	100	14
Ventricle	0.1	1.3	3.0	2.2	0.6	0.6
	20	10	50	25	50	13

TABLE III

EFFECT OF ACTIVATOR ON CYCLIC AMP AND CYCLIC GMP HYDROLYSIS BY F I, F II AND F III OF SINOATRIAL NODE PHOSPHODIESTERASE

The activity of phosphodiesterase was determined at a low suhstrate concentration of 0.4 μM. Assay was performed in the presence of saturated amounts of the activator and 20 μM of Ca^{2+}.

	Percent activation	
	Cyclic AMP hydrolysis	Cyclic GMP hydrolysis
F I	200	140
F II	190	365
F III	112	128

activity was not significantly affected by an addition of the activator in the presence of Ca^{2+}. Cyclic GMP hydrolysis by phosphodiesterase F II was stimulated the most markedly (Table III) and the effects of various concentrations of Ca^{2+} or activator on this preparation were investigated further. The effect of the activator on cyclic GMP hydrolysis by phosphodiesterase F II was investigated in the presence of 20 μM $CaCl_2$ (Fig. 2A). Ca^{2+} stimulated cyclic GMP hydrolysis by phosphodiesterase F II above the concentration of 1 μM (Fig. 2B).

The effect of cyclic GMP on cyclic AMP hydrolysis by phosphodiesterase F I and F II of sinoatrial node is shown in Fig. 3. Cyclic GMP was found to stimulate the hydrolysis of cyclic AMP by these enzymes at low concentrations

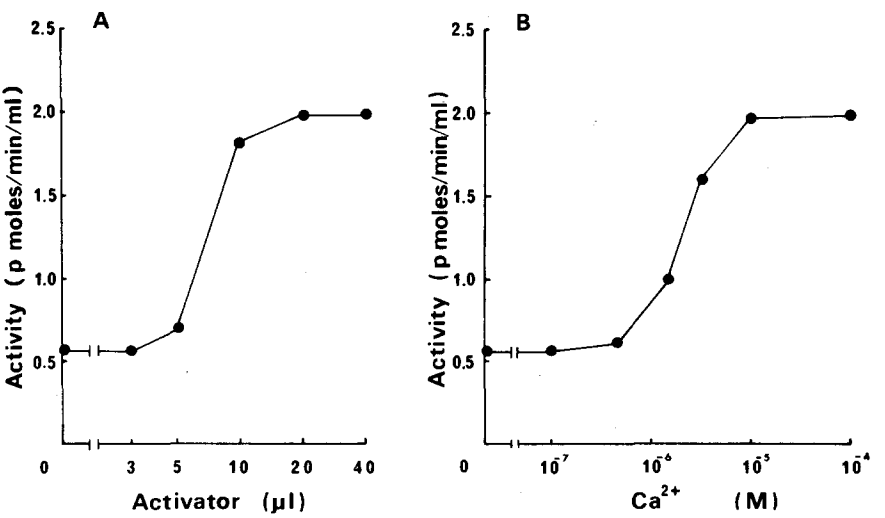

Fig. 2. (A) The effect of the activator on the hydrolysis of cyclic GMP by F II of sinoatrial node phosphodiesterase in the presence of 20 μM $CaCl_2$. The concentration of substrate was 0.4 μM. Activator preparation was dialyzed twice against 10 mM $MgCl_2$ and 0.1 mM EGTA for 24 h. (B) Effect of Ca^{2+} on the hydrolysis of cyclic GMP by a Ca^{2+}-free preparation of the F II fraction of sinoatrial node phosphodiesterase. Assay was performed in the presence of the saturated amounts of the activator. Ca^{2+}-EGTA buffer was used according to the method of Kakiuchi et al. [7].

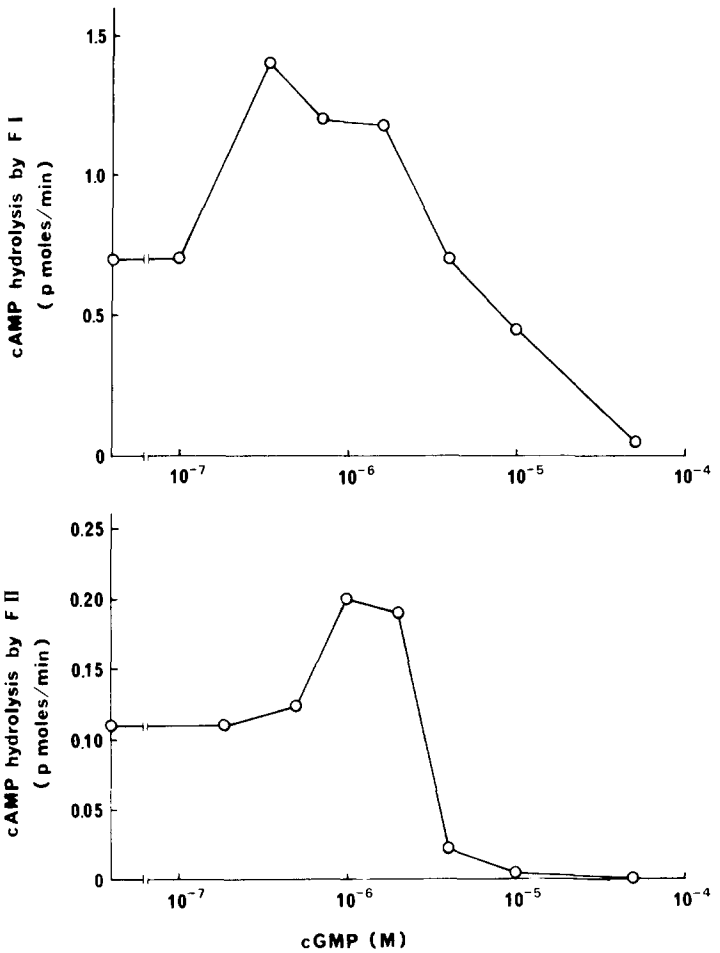

Fig. 3. Effect of cyclic GMP on cyclic AMP hydrolysis by F I and F II fractions of sinoatrial node phosphodiesterase. The concentration of substrate (cyclic AMP) was 0.4 μM.

(below 2 μM) and inhibited cyclic AMP hydrolysis at higher concentrations (above 50 μM). On the other hand, cyclic AMP hydrolytic activity in phosphodiesterase F III was inhibited by micromolar cyclic GMP. When the effect of cyclic AMP on cyclic GMP hydrolysis by phosphodiesterase F I, F II and F III was studied we found that these activities wer inhibited in a dose-dependent manner by cyclic AMP. Cyclic AMP concentrations producing 50% inhibition (I_{50}) of cyclic GMP hydrolysis by phosphodiesterase F I, F II and F III were 6.5, 28 and 10 μM, respectively.

Properties of atrial and ventricular cyclic nucleotide phosphodiesterase

Atria and ventricle also contained three distinct forms of cyclic nucleotide phosphodiesterase similar to those found in the sinoatrial node as determined by DEAE-cellulose chromatography. In atria (Fig. 4), phosphodiesterase F I hydrolyzed almost equally both cyclic AMP and cyclic GMP at a high substrate concentration (100 μM), while phosphodiesterase F II hydrolyzed cyclic GMP

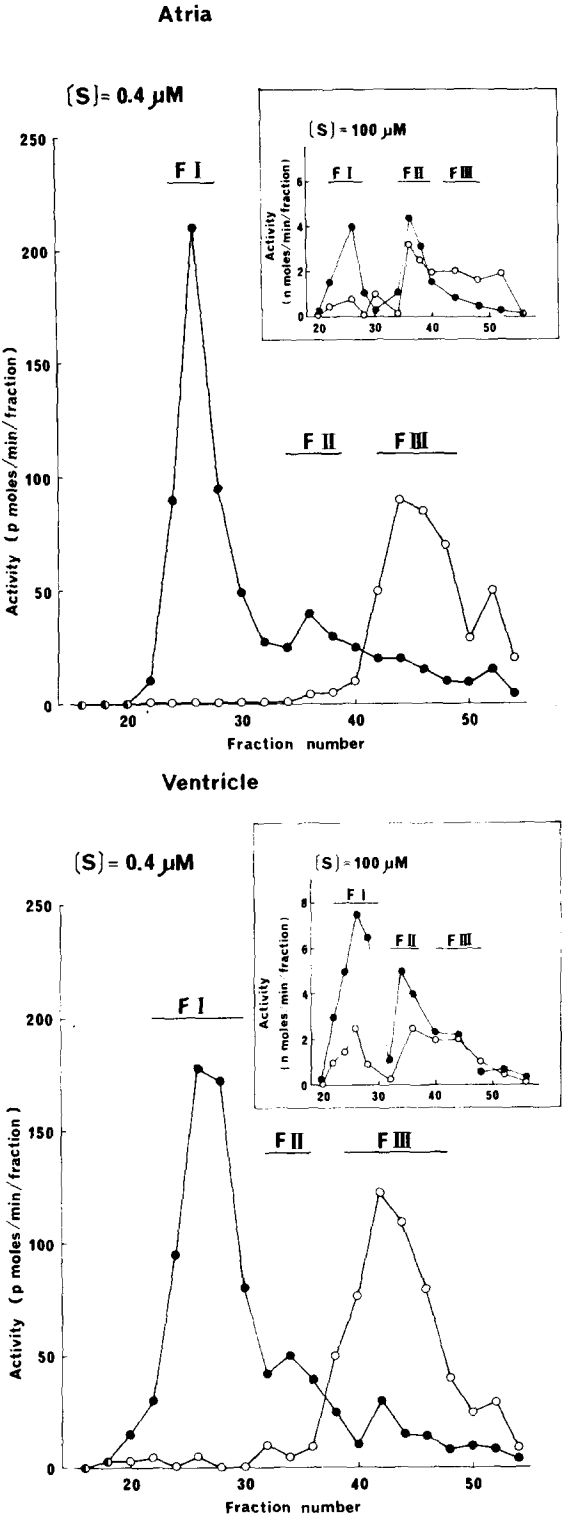

Fig. 4. DEAE-cellulose profile of atria and ventricle phosphodiesterase from 105 000 × *g* supernatant. The gradient was from fraction 20 to 80. Aliquots, 0.2 ml for high substrate (100 μM) or 0.01 ml for low substrate level (0.4 μM), were assayed directly. ○———○, cyclic AMP hydrolysis; ●———●, cyclic GMP hydrolysis.

faster than cyclic AMP at a low substrate concentration (0.4 μM). Phosphodiesterase F III hydrolyzed cyclic AMP faster than cyclic GMP at both 0.4 and 100 μM substrate concentrations. In ventricle (Fig. 4), phosphodiesterase F I and F II hydrolyzed cyclic GMP faster than cyclic AMP at both 0.4 and 100 μM substrate concentrations. Phosphodiesterase F III hydrolyzed cyclic AMP faster than cyclic GMP at a low substrate concentration (0.4 μM), while it hydrolyzed about equally both cyclic AMP and cyclic GMP at a high substrate concentration (100 μM). When the activity was determined at a low substrate concentration (0.4 μM), cyclic AMP hydrolysis by phosphodiesterase F III was higher than that by phosphodiesterase F I in atrial and ventricular preparations, but cyclic AMP hydrolysis by phosphodiesterase F I and F III was about equally in sinoatrial nodal fractions.

Atrial and ventricular phosphodiesterase F II and F III were found to follow abnormal Michaelis-Menten kinetics both for cyclic AMP hydrolysis and for cyclic GMP hydrolysis. In contrast to phosphodiesterase F I of sinoatrial node, phosphodiesterase F I of atria and ventricle displayed abnormal Michaelis-Menten kinetics. These apparent K_m values are summarized in Table II.

The effect of cyclic GMP or cyclic AMP on cyclic AMP or cyclic GMP hydrolysis by phosphodiesterase F I, F II and F III of atria and ventricle were also studied. In contrast to sinoatrial node, cyclic GMP was not stimulatory, but rather inhibited the hydrolysis of cyclic AMP by phosphodiesterase F I and F II of atria and ventricle. The hydrolysis of cyclic AMP by phosphodiesterase F III of atria and ventricle was also inhibited by micromolar cyclic GMP. Cyclic AMP was found to inhibit the hydrolysis of cyclic GMP by phosphodiesterase F I, F II and F III of atria and ventricle as well as sinoatrial node.

Discussion

Studies using partially purified preparation from sinoatrial nodal, atrial and ventricular phosphodiesterase indicate that at least three active cyclic nucleotide phosphodiesterases (F I, F II and F III) are present which are kinetically distinct from each other. There is a pronounced difference in the affinities for substrates between phosphodiesterase F I, F II and F III. All fractions hydrolyzed both cyclic AMP and cyclic GMP but there was no highly specific cyclic GMP phosphodiesterase as in rat liver [5] and no highly specific cyclic AMP phosphodiesterase as in human lymphocytes [9]. The atrial and ventricular elution profiles are quite similar to the profile seen in human blood platelets [10], but the sinoatrial nodal profile is different from profiles of these other tissues.

In our previous report [1], the increase in the content of cyclic AMP in the sinoatrial node after a 1-min exposure to 10^{-5} M noradrenaline was about 2-fold but returned to the control level after a 5-min exposure, whereas adenylate cyclase activity increased and reached a plateau 10 min after exposure; in contrast to sinoatrial node, even after a 5-min exposure to noradrenaline, the cyclic AMP content of the left atrium remained increased. Difference in the time course of cyclic AMP content after exposure to noradrenaline between the sinoatrial node and the left atrium may be attributed to differences in activation in the cyclic nucleotide phosphodiesterase.

Based on experiments carried out in ethyleneglycol-bis(β-aminoethylether)-

N,N'-tetraacetic acid (EGTA)-Ca^{2+} buffer, Teo and Wang [11] reported that a Ca^{2+}-dependent cyclic AMP phosphodiesterase is present in bovine heart. It has been suggested [12] that the Ca^{2+} level during the myocardial concentration cycle fluctuates in the range of 0.1—10 μM. In the present study, a similar range of Ca^{2+} concentrations activated the sinoatrial nodal cyclic nucleotide phosphodiesterase.

Lee et al. [13] and George et al. [14,15] showed that acetylcholine lowered cyclic AMP levels in isolated perfused hearts. George et al. [14,15] and Watanabe and Besch [16] found that acetylcholine treatment elevates cyclic GMP levels in the same preparations. George et al. [17] showed that acetylcholine produced an elevation in atrial cyclic GMP and lowered the levels of cyclic AMP, while isoproterenol produced an elevation in atrial cyclic AMP level, and cyclic GMP levels were not changed. Gardner and Allen [18] showed that acetylcholine produced a time- and dose-dependent increase in cyclic GMP levels and reduced cyclic AMP levels in perfused rat heart. The allosteric activation of cyclic AMP hydrolysis by micromolar amounts of cyclic GMP has been reported by other workers [19,20,5]. In a previous report [10], cyclic AMP hydrolysis by F I of human blood platelet phosphodiesterase was found to be activated by micromolar amounts of cyclic GMP and this activation of cyclic AMP hydrolysis by cyclic GMP was not observed with phosphodiesterase F II and F III. Cyclic AMP hydrolysis by F I and F II of sinoatrial nodal cyclic nucleotide phosphodiesterase was also activated herein by micromolar amounts of cyclic GMP and this activation is not observed with phosphodiesterase F III. These results suggest the possibility that when intracellular levels of cyclic GMP increase up to 1 μM, cyclic AMP hydrolytic activity in sinoatrial node is stimulated by cyclic GMP and this stimulation of cyclic AMP hydrolysis results in a reduction of the cyclic AMP level.

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