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ALTERED DISTRIBUTION AND PROPERTIES OF cAMP-DEPENDENT PROTEIN KINASE ISOZYMES IN SPONTANEOUSLY HYPERTENSIVE RAT AORTA*

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Abstract—cAMP-dependent protein kinase activity was reduced in the cytosol fraction of spontaneously hypertensive rat (SHR) aorta compared to that of the Kyoto Wistar control rat (WKY). Two major peaks, isozymes I and II, of soluble cyclic AMP-dependent protein kinase activity could be separated by DEAE-cellulose chromatography. The distributions of isozymes I and II were 40 and 60%, respectively, in WKY compared to 26 and 74% in SHR. Isozyme I of SHR eluted at a conductance of 2–3 mmhos compared to 5–6 mmhos in WKY. In addition, activity under the peak of isozyme I of SHR was reduced by approximately 55% compared to WKY. The half-life of thermal denaturation of isozyme I at 50° was 21 min in WKY compared to 84 min in SHR. On the other hand, for isozyme II no significant differences were observed between WKY and SHR in elution pattern, total activity under the peak, or thermal denturation of enzyme activity. These data suggest that specific changes had occurred in isozyme I of SHR.

It is generally accepted that the elevation of arterial pressure in essential hypertension is due to an increase in total peripheral resistance. Vascular smooth muscle strips from hypertensive rats show reduced relaxation compared with normotensive control rats after treatment with dibutryl cAMP or with isoproterenol and theophylline [1–3]. This defect in the relaxing ability of vascular smooth muscle cell in hypertensive animals could lead to an increase in vascular tonicity and peripheral resistance. The evidence that implicates a role for cAMP in promoting smooth muscle relaxation has been amply reviewed [4, 5]. In eukaryotic cells, cAMP exerts diverse biologic effects, a number of which are known to be mediated via the activation of protein kinase [6]. cAMP levels in aorta and cultured vascular smooth muscle cells of SHR are lower than in control rats [7–11]. We have shown recently that the reduced levels of cAMP in vascular smooth muscle of SHR could be due to both a decrease in catecholamine-sensitive adenylate cyclase [12, 13] and an increase in membrane-associated cAMP phosphodiesterase [14].

One of the mechanisms through which cAMP exerts its relaxing effect on smooth muscle is mediated through phosphorylation of an endogenous protein of sarcoplasmic reticulum which, in turn, increases energy-dependent Ca^{2+} uptake by the microsomes [15]. It has been shown that microsomal phosphorylation [16] and Ca^{2+} uptake ability [17–19] of vascular smooth muscle of SHR are reduced as compared to WKY. We have shown recently that these changes could be due to decreased levels of cAMP-dependent protein kinase activity in the arterial smooth muscle of SHR [16]. In this communication, we have examined whether decreased cAMP-dependent protein kinase activity of vascular smooth muscle of SHR is due to a decrease in the activity of isozyme I or isozyme II, or both.

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MATERIALS AND METHODS

Sources of chemicals and radiochemicals were the same as described earlier [16]. Adult male SHR and WKY, 12- to 16-weeks-old, were used. The SHR maintained at The University of Iowa are inbred descendents of the hypertensive Wistar strain developed by Okamoto and Aoki [20]. Animals were killed by heart puncture while under ether anesthesia. The aorta was immediately removed, cleaned of loose connective tissue, and placed in homogenizing buffer [0.25 M sucrose in 10 mM Tris–HCl (pH 7.4) containing 1 mM dithiothreitol and 1 mM EDTA] at 4°. The tissue was minced in 10 vol. of the buffer and homogenzied in a polytron (Brinkman) at a rheostat setting of 5.0 for three 10-sec pulses. The homogenate was strained through two layers of cheesecloth and then centrifuged at 105,000 *g* for 90 min. The cytosol (105,000 *g* supernatant fraction) thus obtained was used for the isolation of isozymes, types I and II, using DEAE-cellulose chromatography.

Isolation of cAMP-dependent protein kinase isozymes I and II by DEAE-cellulose chromatography. All the preparative procedures were carried out at 0–5° unless otherwise stated. cAMP-dependent protein kinase isozymes I and II from cytosols of both WKY and SHR aortae were isolated using DEAE-cellulose column chromatography under identical conditions. The cytosolic protein (60–80 mg) was applied to a DEAE-cellulose column (1.2 × 15 cm) pre-equilibrated with 10 mM Tris–HCl (pH 7.4),

1 mM EDTA, and 2 mM β-mercaptoethanol. The column was washed with 50–60 ml of equilibration buffer, and protein kinase isozymes were eluted with a linear gradient of 0–0.6 M NaCl (total gradient 500 ml). Fractions were analyzed for protein kinase activity in the presence and the absence of 1 μM cAMP and for [^{3}H]cAMP binding. The peaks of enzyme activity were pooled and concentrated. Protein was determined by the method of Lowry *et al.* [21] with bovine serum albumin as a standard.

Protein kinase assay. Protein kinase activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone II-AS substrate, as described earlier [16], in the absence or presence of 1 μM cAMP. The reaction was initiated by addition of 10–15 μg protein from various enzyme preparations and incubated at 30°. Reaction was terminated by pipetting 50-μl aliquots of the reaction mixture onto squares (2 cm × 2 cm) of Whatman 3 MM chromatography paper which were dropped immediately into cold 10% trichloroacetic acid (TCA) for removal of residual [γ-^{32}P]ATP, as described by Corbin and Reimann [22]. The reaction was terminated after either 10 or 20 min of incubation. cAMP-dependent protein kinase activity was estimated for 10 min, and the cAMP-independent protein kinase activity for 20 min, to ensure enough ^{32}P incorporation into histone. The reaction was linear for the time of incubation in either case. Specific activity has been expressed as pmoles ^{32}P incorporated per min per mg of enzymic protein.

[^{3}H]*cAMP binding.* The binding of cAMP was measured as described by Gilman [23]. The assay was performed in a total volume of 0.2 ml containing 50 mM sodium acetate (pH 5.0), 1 mM EDTA, 20–40 μg of enzyme protein and 50 nM [^{3}H]cAMP (~32 Ci/mmole). After 3–4 hr incubation at 4°, the reaction was stopped by the addition of 2 ml of the same buffer, and the mixture was then immediately filtered through a millipore filter which had been premoistened with cold buffer. The tube and the filter were rinsed with 5 ml of cold buffer. The filter containing the protein-bound [^{3}H]cAMP was placed in toluene based scintillant, and the radioactivity was determined in a liquid scintillation counter. The results were expressed as pmoles cAMP bound per assay tube for elution profiles. Blank values (no enzymes) were subtracted from the experimental values.

RESULTS

Linearity with respect to time and protein concentration. cAMP-dependent and independent protein kinase activities of isozymes I and II were linear up to 15 μg protein/assay (Fig. 1). The reaction rate was linear at 30° up to 40 min in the absence of cAMP and up to 20 min in the presence of 1 μM cAMP. The following experiments were performed under conditions of linearity both with respect to time of incubation and protein concentration.

Distribution of isozymes I and II in WKY and SHR aortae. cAMP-dependent protein kinase activity was reduced significantly ($P < 0.05$) in the cytosol fraction of SHR aorta, compared to WKY. Two distinct peaks of protein kinase activity were isolated on

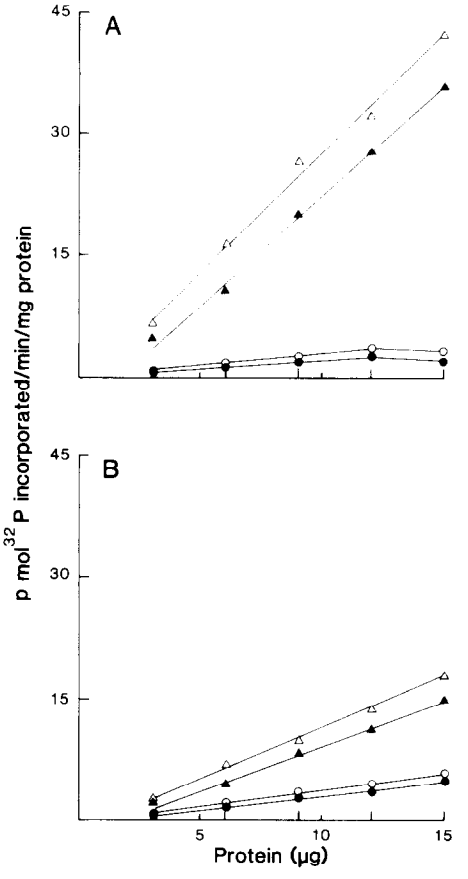

Fig. 1. Linearity of protein kinase activity with respect to protein concentration. (A) isozyme I; (B) isozyme II (△,○ WKY; ▲,● SHR). Activity was measured as described in Materials and Methods with (△,▲) or without (○,●) 1 μM cAMP. Protein was from 2 to 15 μg/tube. Incubation time was 10 min for cAMP-dependent and 20 min for cAMP-independent enzyme activity. Results are means of two separate experiments in duplicate.

DEAE-cellulose chromatography from WKY and SHR aortae soluble fractions. The isozyme I of SHR eluted from the column at a conductance of 2–3 mmhos, whereas WKY isozyme I eluted at a conductance of 5–6 mmhos. On the other hand, isozyme II of both SHR and WKY eluted at a conductance of 11–12 mmhos. Furthermore, the major amount of isozyme I of SHR eluted in 5 fractions as compared to 15–16 fractions in WKY. No difference between the eluting patterns of WKY and SHR isozyme II was observed. Binding of cAMP corresponded to the peaks of cAMP-dependent protein kinases (Fig. 2). The relative activity distribution of the two isozymes was determined by adding the activities in the column fractions that comprised each major peak and calculating percentages of I and II. In the control group, the distribution of isozymes I and II was 40.2 and 59.8%, respectively, compared to 26.0 and 74.0% in SHR (mean of two experiments). The enzyme activity distribution was also calculated by multiplying the specific activity and the total protein eluted under each peak. The results were similar to those obtained by addition of enzyme units in each

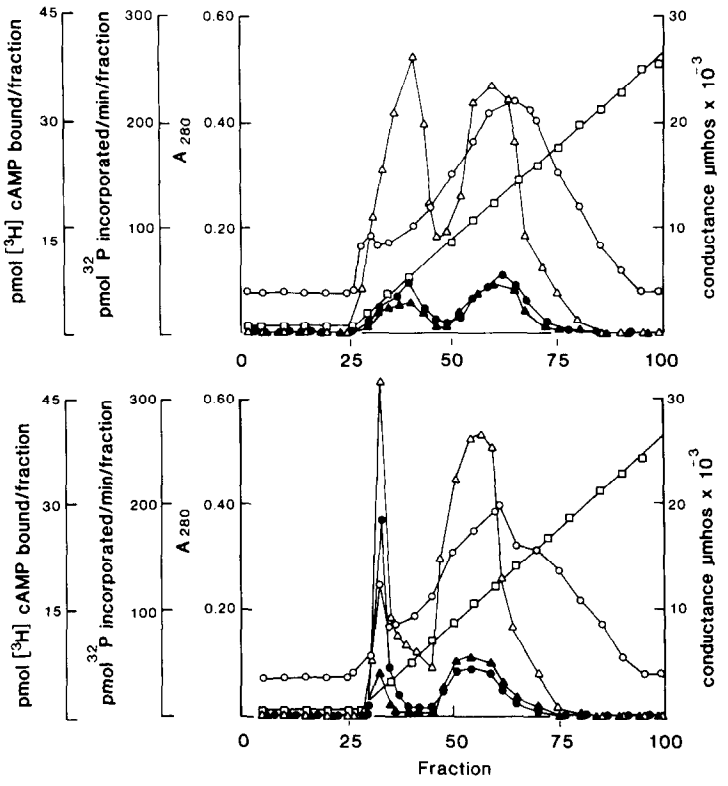

Fig. 2. DEAE-cellulose chromatography of cAMP-dependent protein kinase in the 105,000 *g* supernatant fraction of WKY (upper panel) and SHR (lower panel) rat aortae. The same amounts of protein obtained from the 105,000 *g* supernatant fractions of WKY and SHR aortae were applied to two identical columns. The preparation of partially purified cAMP-dependent protein kinase is described in Materials and Methods. The fractions were assayed for protein kinase in the presence (△—△) or absence (▲—▲) of 1 μM cAMP. They were also assayed for [^{3}H]cAMP binding (●—●) protein (○—○), and conductance (□—□). Each fraction was assayed for cAMP-dependent protein kinase activity, and total enzyme units in each fraction were calculated and summed up for the determination of percentage of distribution of isozymes I and II in WKY and SHR. One enzyme unit is equivalent to the incorporation of 1 pmole ^{32}P into histone per min at 30°.

Table 1. Apparent kinetic constants of cAMP-dependent protein kinase isozymes I and II from WKY and SHR aortae*

Kinetic constants	Isozyme I		Isozyme II	
	WKY	SHR	WKY	SHR
ATP				
K_m (μM)	100.3	97.5	117.6	108.0
V_{max}	3750	2664	1517	1023
Mg^{2+}				
K_m (mM)	1.5	1.2	1.4	1.5
V_{max}	4440	2970	1950	1500
cAMP				
K_m (nM)	18.0	22.0	15.0	16.0
V_{max}	3750	2850	1570	1210

* Assays were carried out as described in Materials and Methods using various substrate concentrations in the presence of 1 μM cAMP. Enzyme protein was approximately 10 μg/assay tube. The kinetic constants were calculated by double-reciprocal Lineweaver–Burk plots drawn by linear regression. V_{max} is expressed as pmoles ^{32}P transferred per min per mg protein. Each value is the mean from two separate experiments carried out in triplicate.

fraction. Comparison between WKY and SHR total enzyme activities under the peaks showed that isozyme I of SHR was decreased by approximately 55% compared to WKY. On the one hand, the total activity under the peak of isozyme II of SHR was reduced by 13% compared to WKY. The protein, eluted for isozyme I of SHR, was decreased by 14% compared to WKY. On the other hand, for isozyme II the amount of protein that eluted was 23% higher in SHR than in WKY. These data suggest that the isozyme II of SHR may have been differentially contaminated with non-specific proteins, and that a specific decrease occurred in isozyme I of SHR which could account for the decreased cAMP-dependent protein kinase activity found in the soluble fraction of SHR aorta. In rat aorta, isozyme II appeared to be the predominant enzyme. Although isozyme I constituted a lesser proportion of the total kinase activity, its reaction velocity was 2 to 2.5 times higher than that of isozyme II, both in WKY and SHR (Table 1).

Properties of protein kinase isozymes. The apparent kinetic constants, of pooled fractions of isozymes

Table 2. Dissociation of protein kinase isozymes I and II by salt and histone*

Addition	Time of incubation (min)	Ratio of −cAMP/+cAMP			
		Isozyme I		Isozyme II	
		WKY	SHR	WKY	SHR
None	0	0.19	0.18	0.39	0.39
	15	0.15	0.15	0.40	0.40
	30	0.14	0.13	0.40	0.40
0.5 M NaCl	0	0.18	0.19	0.37	0.37
	15	0.33	0.34	0.40	0.38
	30	0.36	0.37	0.39	0.38
Histone, 700 μg/ml	0	0.18	0.18	0.33	0.41
	15	0.91	0.96	0.34	0.36
	30	1.0	1.0	0.36	0.38

* At zero time NaCl, histone or buffer was added. The samples were incubated at 30° for the indicated times. At the given intervals, a 25-μl aliquot was added to 75 μl of reaction mixture to measure enzyme activity in the presence and absence of 1 μM cAMP. The reaction was incubated for 10 min at 30° and terminated by the addition of 10% trichloroacetic acid. Values are expressed as the activity in the absence of cAMP divided by the activity in the presence of cAMP. Each value is the average of two different experiments.

I and II isolated by DEAE-cellulose chromatography (Fig. 2), for ATP and Mg^{2+} were calculated by Lineweaver–Burk plots. The relations drawn by regression analysis were linear. The data in Table 1 show that the apparent K_m values for cAMP, ATP and Mg^{2+} for isozymes I and II of SHR were comparable to those of WKY. The total activity, as well as specific activity, of pooled fractions was reduced (V_{max} in Table 1) compared to WKY. The specific activity of isozyme II pooled fractions was also decreased in SHR compared to WKY (V_{max} in Table 1). The 25% decrease in V_{max} coincided with the 23% increase in protein eluted under peak II of SHR. It is, therefore, likely that the decrease in specific activity of isozyme II in SHR was due to a non-specific contamination by other proteins.

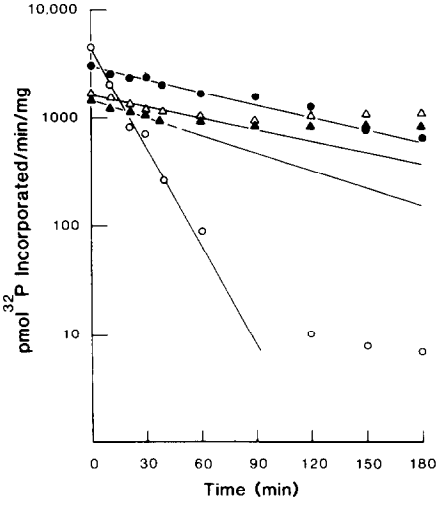

Fig. 3. Thermal inactivation of isozyme I (○,●) and isozyme II (△,▲) of control (○,△) and SHR (●,▲) aortae. Enzyme preparations (1 mg/ml) were incubated at 50° for the times indicated, then cooled to 4°, and their kinase activities assayed in the presence of 1 μM cAMP using 15 μg protein/assay.

The substrate specificities of isozymes I and II of WKY and SHR were tested using various protein substrates in the presence or absence of 1 μM cAMP. The substrate specificities for isozymes I and II were in the order of histone II AS > histone III S > β-casein > histone VIII S > protamine. For all the substrates tested, the enzyme activities of isozymes I and II were lower in SHR than in WKY.

As defined by Corbin *et al.* [24], isozyme I protein kinase is rapidly dissociated in the presence of 0.5 M salt or histone, whereas the regulatory and catalytic subunits of isozyme II are not dissociated even after 30 min of preincubation with NaCl or histone. The effect of preincubating isozymes I and II with either 0.5 M NaCl or 700 μg/ml histone was investigated (Table 2). The ratio −cAMP/+cAMP was measured at different periods of incubation up to 30 min. Isozyme I dissociated in the presence of NaCl and histone. However, no difference between SHR and WKY was observed in the extent of dissociation of isozyme I.

Thermostability of protein kinase isozymes. Thermostability of enzyme activity was examined by prior incubation of protein kinase isozymes for 3 hr at 22°, 30°, 40°, and 50°, and then assay of the enzyme activity in the presence of 1 μM cAMP. Isozymes I and II showed a gradual decrease in the enzyme activity with increasing temperatures from 0° to 40°; however, raising the temperature from 40° to 50° resulted in a significantly greater decrease in WKY isozyme I activity compared to SHR isozyme I. At 22° and 30° there were no differences between SHR and WKY in the extent of enzyme inactivation. To test whether differences exist in the rate of inactivation of WKY and SHR isozymes, the half-life of thermal denaturation was calculated at 50°. The data are given in Fig. 3. The half-life of thermal denaturation of isozyme I was about 21 and 84 min, and of isozyme II 186 and 140 min, for WKY and SHR respectively (average of four determinations). These data suggest that significant alterations had appeared in the thermostability of isozyme I of SHR. Furthermore, isozyme II was more stable than isozyme I in both SHR and WKY.

DISCUSSION

Observations made in this [16] and other [25] laboratories have shown that the specific activity of cAMP-dependent protein kinase is reduced significantly in the cytosol fraction of SHR aorta compared to WKY. The data presented in this paper demonstrate a 50% decrease in total cAMP-dependent protein kinase activity in the isozyme I of SHR compared to WKY. There were no significant differences between SHR and WKY in the total cAMP-dependent protein kinase activity of isozyme II. These results suggest that in SHR vascular smooth

muscle the reduced cAMP-dependent protein kinase activity is due to a decrease in isozyme I activity. In pathological conditions, it has been observed that isozyme I is more susceptible than isozyme II to alterations in distribution. Alterations in distribution of isozyme I in association with cardiac hypertrophy have been reported [26, 27]. However, in these studies the isozyme I was increased under pathological conditions. The physiological significance of isozymes I and II in vascular smooth muscle is not known. However, substantial evidence exists linking cAMP and relaxation in vascular smooth muscle [5, 28]. Specifically, relaxation of vascular smooth muscle strips, caused by β-adrenergic stimulation, various phosphodiesterase inhibitors, or dibutyryl cAMP, is correlated with increases in intracellular cAMP levels [29–31]. Vascular strips of aorta from spontaneously hypertensive rats have been shown to be less responsive than WKY strips to dibutyryl cAMP-induced relaxation [32]. Since we observed a specific decrease only in isozyme I of SHR (compared to WKY), this circumstantial evidence would tend to suggest that isozyme I of cAMP-dependent protein kinase may be of greater significance in the regulation of smooth muscle relaxation in normal and pathological conditions.

Most tissues examined contain at least two isozymes of cAMP-dependent protein kinases that can be separated by DEAE-cellulose chromatography. Isozyme I elutes at lower conductance than isozyme II [24]. Both kinases are thought to contain the same catalytic subunit but different regulatory subunits [33]. Therefore, it is possible that any differences in the properties of either protein kinase I or II are due to a change in the regulatory subunit. Data shown here indicate that the conductance at which isozyme I of SHR eluted was reduced considerably compared to WKY. Similarly the half-life of thermal denaturation of isozyme I of SHR was increased to 84 min compared to 21 min in WKY. Similar to these observations, an increase in $T_{\frac{1}{2}}$ of thermal denaturation of cAMP-dependent protein kinase has been observed in a cancerous cell line by Hochman *et al.* [34]. These authors attributed the change in thermostability to a genetic modification in the R-subunit of the cancerous tissue. It is possible that in SHR an alteration in the R-subunit of isozyme I has occurred. Further characterization of the nature of subunit interaction of isozyme I in SHR and WKY vascular smooth muscle needs to be investigated. Recombiant experiments between the subunits isolated from isozyme I of SHR and WKY aortae would help determine if the alterations in the thermostability and clution profile are due to a defect in the R-subunit of isozyme I in SHR.

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