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Calcium Fluxes, Calcium Binding, and Adenosine Cyclic 3′,5′-Monophosphate-Dependent Protein Kinase Activity in the Aorta of Spontaneously Hypertensive and Kyoto Wistar Normotensive Rats

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SUMMARY

BHALLA, R. C., WEBB, R. C., SINGH, D., ASHLEY, T. & BROCK, T. (1978) Calcium fluxes, calcium binding, and adenosine cyclic 3′,5′-monophosphate-dependent protein kinase activity in the aorta of spontaneously hypertensive and Kyoto Wistar normotensive rats. *Mol. Pharmacol.*, 14, 468–477.

Spontaneously hypertensive rats and Kyoto Wistar normotensive rats were compared with respect to calcium fluxes in aortic strips, membrane phosphorylation and calcium uptake by microsomes, and cyclic AMP-dependent protein kinase activity in tissue extracts of aorta. The aortic strips of hypertensive rats accumulated more ^{45}Ca than those of normotensive animals. The calcium content of the aorta increased with age of the animals from 30 days to 1 year and was consistently higher in hypertensive than in normotensive rats. Calcium uptake by the aortic microsomes was significantly decreased in hypertensive ($p < 0.05$) compared with normotensive rats in the presence and absence of 1 μM cyclic AMP, as was cyclic AMP-dependent phosphorylation of microsomes. Cyclic AMP-dependent and -independent protein kinase activity in the 27,000 × g supernatant of the aortas of 12–16-week-old hypertensive rats was reduced by 42% and 34%, respectively, using histone as a substrate. Differences both in calcium uptake by microsomes treated with cyclic AMP and in cyclic AMP-dependent protein kinase activity in the tissue extracts were evident at 30 days of age. These observations reflect the importance of impaired membrane phosphorylation in the pathogenesis of hypertension.

INTRODUCTION

In essential hypertension, increased peripheral resistance and elevated arterial pressure seem causally related to an increase in the tonicity of vascular smooth muscle. As in the case of skeletal muscle, the amount of calcium ion present at the contractile apparatus appears to regulate the tone, or contractile state, of smooth muscle (1).

Two possible mechanisms can be considered in the regulation of the availability of calcium in smooth muscle cell cytoplasm: the movement of calcium across the plasma membrane and the sequestration and release of calcium by intracellular storage sites. Abnormalities in each of these mechanisms have been implicated in the pathogenesis of hypertension. Regarding the first mechanism, an increase in total calcium content of blood vessels from hypertensive

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rats has been reported (2–4). Studies have also shown increased membrane permeability to extracellular calcium (5) and a generalized increase in ion permeability or membrane "leakiness" (6–8) in the hypertensive state. Concerning the second mechanism, our laboratory (9) and others (10, 11) have reported significantly reduced calcium uptake by microsomal vesicles of spontaneously hypertensive rats. Reversible phosphorylation of microsomal membranes by cyclic AMP-dependent protein kinase has been postulated as an important process in the regulation of microsomal Ca^{++} transport in cardiac and skeletal (12) and vascular smooth muscle (13–16). Abnormalities in cyclic AMP metabolism have previously been related to intracellular regulation of vascular tone in spontaneously hypertensive rats (17–25). The abnormality of cyclic AMP metabolism might express itself via this protein kinase-controlled microsomal calcium sequestration.

In the present communication we report results of experiments designed to test the relative roles of these two pathways in the aortas of Kyoto Wistar spontaneously hypertensive and normotensive rats. We have observed an increase in calcium uptake in aortic smooth muscle strips and a decrease in the basal and cyclic AMP-stimulated calcium-sequestering ability of aortic microsomes from hypertensive rats. The latter observation, together with deficient microsomal phosphorylation and cyclic AMP-dependent and -independent protein kinase activity observed in the hypertensive animals, suggests a mechanism that would lead to an increased cytoplasmic calcium level in the aorta of hypertensive animals, and hence to increased smooth muscle tone.

MATERIALS AND METHODS

Adult male and female Kyoto Wistar spontaneously hypertensive rats, Kyoto Wistar normotensive rats 12–16 weeks old, and normotensive Sprague-Dawley rats weighing 200–350 g were used. Young, prehypertensive rats were used at age 28–32 days. The spontaneously hypertensive rats maintained at the University of Iowa are inbred descendants of the hypertensive Wistar strain developed by Okamoto and Aoki (25). The hypertensive rats used in this study correspond to the F_{29}–F_{35} generations from the original pairing; the normotensive rats correspond to the F_8–F_{12} generations. The control rats were raised under conditions identical with those used for the hypertensive animals. Preoperative systolic blood pressures were determined in the unanesthetized state by the tail plethysmographic method, using an automated cuff inflator-pulse-reading system manufactured by Technilab Instruments. ^{45}Ca (about 11 mCi/mg), [γ-^{32}P]ATP (more than 10 Ci/mmole), cyclic [^3H]AMP (about 16 mCi/mmole), and Aquasol were obtained from New England Nuclear. Soluble cyclic AMP-dependent protein kinase (beef heart), cyclic AMP, ATP, DEAE-cellulose, bovine serum albumin, and histone, type IIA, were purchased from Sigma Chemical Company. Filters (0.45 μm, 25 mm in diameter) were obtained from Millipore Corporation. Dioxane and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene were supplied by Mallinckrodt, Inc., and 2,5-diphenyloxazole was obtained from Fisher Chemical Company.

Preparation of subcellular fractions. Adult animals were stunned and killed by cervical dislocation or decapitation, and young animals, under ether anesthesia. The aortas were immediately removed, trimmed of adherent fat and loose connective tissue, and rinsed in cold homogenizing buffer (0.25 M sucrose in 20 mM Tris-HCl, pH 7.4). The subcellular fractions were prepared according to Fitzpatrick *et al.* (26), as described earlier (9).

Calcium accumulation by vascular strips. Thoracic aortas were cut into helical strips and placed in a beaker containing Tris-buffered physiological salt solution containing NaCl, 139 mM; KCl, 4.1 mM; $MgCl_2$, 2.1 mM; dextrose, 10 mM; Tris-HCl, 20 mM; and $CaCl_2$, 1.53 mM, at pH 7.4. The solution was bubbled with 95% O_2–5% CO_2, and the temperature was maintained at 37°. The vascular strips were blotted, weighed, mounted on Pyrex glass rods, and then incubated for 2–3 hr in PSS.[3] At the end of

[3] The abbreviations used are: PSS, Tris-buffered physiological salt solution, whose composition is given in the text; TCA, trichloracetic acid.

this initial incubation period, the strips were incubated in PSS containing ^{45}Ca (0.4 μCi/ml). The strips were transferred at timed intervals to Ca^{++}-free PSS containing 10 mM lanthanum chloride and incubated for 45 min. Lanthanum is thought to inhibit the loss of calcium ions from within the smooth muscle cell while displacing the membrane-bound and extracellular pool of calcium ions (27, 28). The strips were then cut from the Pyrex rods and dissolved in 0.6 ml of Protosol (New England Nuclear), and the radioactivity was determined by liquid scintillation spectrometry.

Calcium efflux from vascular strips. Vascular strips prepared from thoracic aorta were first incubated for 1 hr in PSS at room temperature, then for 1 hr at 37° in PSS containing ^{45}Ca, 0.15 μCi/ml. Each strip was tied with surgical thread to a Pyrex glass rod at a tension just sufficient to extend the surface of the strip fully. All solutions were bubbled with 95% O_2–5% CO_2. At the end of the desired incubation interval the tissue was removed from the radioactive solution and dipped rapidly into two sequential beakers of nonradioactive PSS. The tissues were then transferred through a series of 2-ml aliquots of PSS at 37° at intervals of 5 min, up to a maximum of 120 min. At the end of the final washout period, the tissues were removed, blotted, and dissolved in 0.6 ml of Protosol at 65° overnight to determine radioactivity. The desaturation curves express the decline of tissue ^{45}Ca concentration with time, determined both from the ^{45}Ca present in washout samples and from the ^{45}Ca remaining in the muscle after the 120-min washout period. This type of plot can be used to illustrate alterations in ^{45}Ca loss (29, 30). The parameter used to describe the rate of exchange of calcium in the tissue is the constant k, equal to the reciprocal of the time required for one ion pool to decrease from an original value x_0 to x_0 divided by e, the natural logarithm base. Derived from the slope of the efflux line, k is proportional to the rate of turnover of calcium in the compartment that it describes (31).

Total tissue calcium. Aortas were removed from arch to bifurcation and cleaned of adhering blood and loose connective tissue. Tissue was processed for determination of total tissue calcium by a slight variation of the method of Altura and Altura (32). Vascular strips were blotted on ash-free filter paper, and the net weight was determined in previously weighed porcelain crucibles. The tissue was dried overnight at 110°, and its dry weight was determined. The tissue was then digested in 0.1 ml of concentrated HCl for 3–4 hr at 110° and ashed at 410–425° for 24 hr. The sample was suspended in 1.0 ml of concentrated HCl and 4.0 ml of water and diluted 1:2 with 2% $LaCl_3$ to prevent interference by phosphorus. The calcium content was determined by atomic absorption spectrophotometry (Perkin-Elmer atomic absorption spectrophotometer, model 303).

Ca^{++} uptake by subcellular fractions. The following mixture was utilized for Ca^{++} uptake measurements: 100 mM KCl, 7.5 mM oxalate, 2 mM $MgCl_2$, 2 mM ATP, 10 μM $CaCl_2$, 0.2 μCi of $^{45}Ca^{++}$, 15 mM NaN_3, 20 mM Tris-HCl (pH 7.4), and 50–100 μg of protein in a total volume of 1.0 ml. The reaction was terminated by passing the reaction mixture quickly through Millipore cellulose acetate filters (pore size, 0.45 μm). The filters were then dried, dissolved in Bray's solution, and counted for radioactivity.

Measurement of protein kinase. Histone was used as the substrate for protein kinase. Activity was measured in 0.11 ml containing 50 mM acetate (pH 6.0), 1.8 mM NaF, 3.7 mM theophylline, 0.22 mg of histone, 22 mM $MgCl_2$, 0.1 mM ATP ($1–2 \times 10^5$ cpm), and 1 μM cyclic AMP when added. The histone and cyclic AMP were added in rapid succession, and the reaction was immediately initiated by the addition of [γ-^{32}P]ATP–$MgCl_2$. After incubation at 30° for 7 min, 3 ml of cold 20% TCA were added with mixing, and the tubes were allowed to stand for at least 5 min in ice. Samples were centrifuged, the supernatant liquid was decanted, and the pellets were resuspended twice in 2 ml of 20% TCA by the procedure of Kuo and Greengard (33). The washed precipitate was dissolved in 0.20 ml of 1 N sodium hydroxide, and a 0.10-ml aliquot was counted in 10 ml of Bray's solution. Enzyme and histone blanks were suffi-

ciently low to justify omission of the intermediate solubilization and reprecipitation. Incorporation of ^{32}P into histone at 30° was linear for 20 min and proportional to kinase concentration up to 200 μg of protein.

Phosphorylation of microsomal protein. Phosphorylation of microsomes was carried out at 30° in 200 μl of 20 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 1–2 μCi of [γ-^{32}P]ATP, 1 μM cyclic AMP, and about 50 μg of microsomal protein. The reaction was initiated by the addition of protein and terminated by the addition of 4.0 ml of 20% (w/v) ice-cold TCA, followed immediately by 0.2 ml each of 0.2 M KH_2PO_4 and 0.63% bovine serum albumin. After remaining on ice for 10 min, the samples were centrifuged for 10 min at 1500 × *g*. The supernatant was decanted, and the pellets were dissolved in 0.1 ml of 0.5 N NaOH at room temperature. Then 4.0 ml of 20% TCA were added, the centrifugation was repeated, and the pellets were washed with 2.0 ml of 20% TCA three more times. The final pellets were dissolved in 0.2 ml of 1.0 N NaOH, and 0.1-ml aliquots were transferred to scintillation vials containing 10 ml of Aquasol for determination of radioactivity.

Determination of cyclic AMP levels. Tissues were preincubated in Eagle's minimal essential medium for 20 min in the presence of 1 mM theophylline. After termination of the 10-min experimental incubation the tissues were immediately frozen in liquid nitrogen and homogenized with a Polytron tissue disintegrator in 6% TCA. After centrifugation at 1500 × *g* for 20 min, the supernatant was collected for assay of cyclic AMP by the method of Walton and Garren (34) with minor modifications (35). This method employs cyclic AMP-binding protein as receptor, cyclic [^3H]AMP as ligand, and Millipore filtration to separate the bound from the free nucleotide. The precipitate was used for protein and specific activity determinations.

RESULTS

The average blood pressure of adult spontaneously hypertensive rats was 186 ± 2 mm Hg (N = 246), compared with 134 ± 2 mm Hg (N = 238) for normotensive Wistar rats; the difference is significant ($p < 0.05$). The average blood pressures of young normotensive and hypertensive rats were 119 ± 1.8 (N = 20) and 123 ± 1.4 (N = 21) mm Hg, respectively. The blood pressure of Sprague-Dawley rats was 135 ± 4.3 mm Hg (N = 9).

Characterization of microsomal fraction. Electron micrographs of the 100,000 × *g* pellet (microsomal fraction) showed that this fraction consisted of vesicular structures. Most vesicles consisted of smooth membranes, but occasional rough membrane vesicles were encountered. No intact mitochondrial fragments could be identified in these preparations. Biochemical characterization of subcellular fractions as reported earlier (9) was carried out by determination of cytochrome oxidase and succinate dehydrogenase. The activity of these mitochondrial enzyme markers per milligram of protein in this fraction was less than 10% of that in the mitochondrial fraction (8700 × *g*) pellet). In addition, mitochondrial contamination was also ruled out by the observation that azide had no effect on calcium sequestration by the microsomal fractions in the presence of oxalate. The yield of microsomal protein was about 1.62 mg/g of vascular smooth muscle tissue, wet weight, and there was no significant difference in the yield between hypertensive and normotensive rats.

Calcium accumulation by vascular smooth muscle strips. The vascular smooth muscle strips prepared from aortas of hypertensive rats accumulated significantly ($p < 0.05$) more ^{45}Ca than normotensive controls at 2.5- and 5-min incubation intervals. In experiments utilizing Sprague-Dawley rats as supplemental controls, it was found that calcium accumulation by aortic strips of hypertensive rats was significantly higher at 5, 15, and 20 min of incubation. Aortic strips from normotensive Wistar rats consistently accumulated more calcium than those from Sprague-Dawley rats (Fig. 1).

Calcium efflux from vascular smooth muscle strips. Figure 2 shows the efflux of ^{45}Ca from aortic strips in Ca^{++}-free PSS and PSS containing 1.8 mM $CaCl_2$. Each of these curves is analyzed most simply by

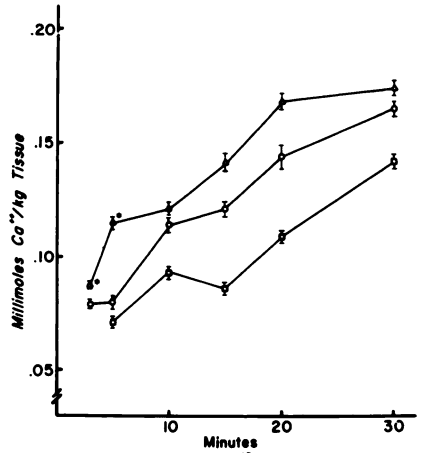

FIG. 1. *Time course of ^{45}Ca accumulation by aortic strips using lanthanum method*

The procedure for measuring ^{45}Ca accumulation is described under MATERIALS AND METHODS. Values are means ± standard errors of eight or nine determinations. △——△, spontaneously hypertensive; ○——○, normotensive Kyoto Wistar; □——□, normotensive Sprague-Dawley rats.

* Significantly different ($p < 0.05$) from normotensive Kyoto Wistar rats. The values for hypertensive rats were also significantly different ($p < 0.05$) from those for normotensive Sprague-Dawley rats at the 5-, 15-, and 20-min intervals.

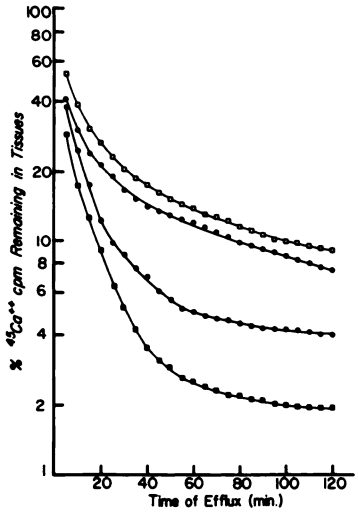

FIG. 2. *Time course of ^{45}Ca efflux from aortic strips in Ca^{++}-free PSS (open symbols) and PSS containing 1.8 mM $CaCl_2$ (solid symbols)*

The procedure for measuring ^{45}Ca efflux is described in MATERIALS AND METHODS. The data are representative of four different experiments. □——□ and ■——■, spontaneously hypertensive; ○——○ and ●——●, normotensive Kyoto Wistar rats.

resolution into two components. One component is described by making a least-squares fit of the data for the final 50 min of efflux. If the line so generated is extrapolated to zero time, and the values on this line are subtracted from experimental values, a steep straight line describing efflux from the other, more rapidly exchanging compartment results. In calcium-free PSS, ^{45}Ca efflux from the rapidly exchanging component proceeded much more slowly for both normotensive and hypertensive rats. In PSS containing 1.8 mM $CaCl_2$, the component of the curve pertaining to the slowly exchanging pool reveals a marked difference between the normotensive and hypertensive animals. In this pool the constant k equals 0.0024 min^{-1} for normotensive and 0.0060 min^{-1} for hypertensive rats, suggesting a more rapid exchange in the latter. When efflux experiments were performed using aortic strips from prehypertensive and normotensive rats (age, 30 days), no differences in ^{45}Ca efflux could be detected in the absence or presence of 1.8 mM $CaCl_2$. In addition, both groups showed indistinguishable rates of exchange.

Tissue calcium content. The total tissue calcium content of the aortas of spontaneously hypertensive and normotensive rats of various ages is shown in Fig. 3. The calcium content dramatically increased with age of the animals from 31 days to 1 year. Aortas from 1-year-old hypertensive rats contained significantly more ($p < 0.05$)

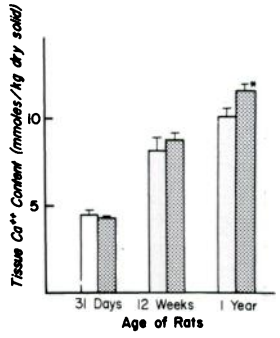

FIG. 3. *Total tissue calcium content at different ages of aortas from spontaneously hypertensive (stippled) and normotensive Kyoto Wistar rats (open bars)*

Values are means ± standard errors of 9–12 determinations.

* Significant difference ($p < 0.05$).

calcium than those from normotensive animals. In 12-week-old animals this imbalance appeared consistent, although at an insignificant level. However, in the prehypertensive stage (28–32 days old), there were no differences between the two groups.

Cyclic AMP-stimulated phosphorylation of microsomes. The time course of microsomal protein phosphorylation is shown in Fig. 4. In the absence of cyclic AMP, a small but statistically significant difference ($p < 0.05$) was found between spontaneously hypertensive and normotensive rats. Addition of 1 μM cyclic AMP stimulated phosphorylation over the basal levels in both groups. However, the incorporation of ^{32}P in microsomes from hypertensive rats was significantly lower ($p < 0.05$) than in normotensive animals at all incubation intervals.

Calcium uptake by aortic microsomes of rats treated with cyclic AMP. The time course of energy-dependent calcium uptake by aortic microsomes in the presence and absence of 1 μM cyclic AMP is shown in Fig. 5. At all incubation intervals tested,

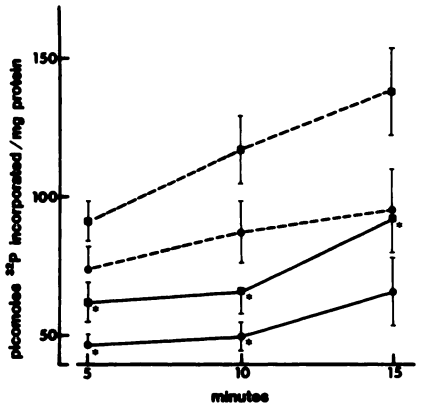

FIG. 4. *Time course of microsomal phosphorylation*

Aortic microsomes from spontaneously hypertensive (——) and normotensive Kyoto Wistar (- - -) rats were incubated under standard assay conditions, described under MATERIALS AND METHODS, in the presence (■) and absence (●) of 1 μM cyclic AMP. Each value is the mean ± standard error of five determinations. For each experiment microsomes were prepared from the aortas of 10–12 rats.

* Significantly different from the respective normotensive control value ($p < 0.05$).

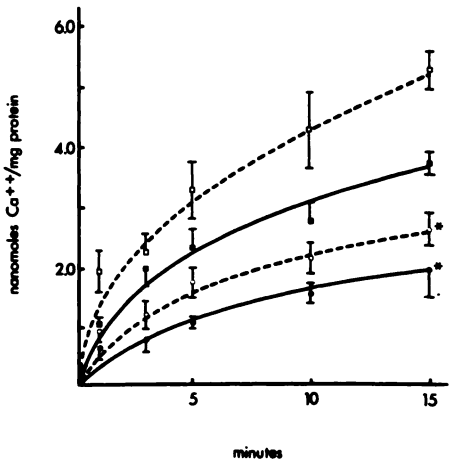

FIG. 5. *Time course of calcium uptake by aortic microsomes in the presence* (- - -) *and absence* (——) *of 1 μM cyclic AMP*

Microsomes from aortic homogenates of (spontaneously hypertensive (○, ●) and normotensive Kyoto Wistar rats (□, ■) were first incubated for 15 min in the reaction mixture for measuring phosphorylation (see MATERIALS AND METHODS), except that nonradioactive ATP was used. Then an aliquot of the reaction medium was added to the assay mixture (described in the text) for measuring calcium uptake. The reaction was terminated by passing the mixtures through Millipore filters, and their radioactivity was determined. Each value is the mean ± standard error of three determinations. For each experiment microsomes were prepared from the aortas of 10–12 rats.

* Significantly different from normotensive control ($p < 0.05$).

calcium uptake was reduced in spontaneously hypertensive rats; significant differences ($p < 0.05$) were observed at the 15-min incubation interval. The ability of the aortic microsomes to sequester calcium was studied in the prehypertensive stage (30 days of age) in both groups of animals. Whereas no significant change was seen in the absence of cyclic AMP (Table 1), the microsomes from spontaneously hypertensive rats showed significantly reduced ($p < 0.05$) calcium uptake in the presence of cyclic AMP.

Cyclic AMP levels and cyclic AMP-dependent and -independent protein kinase activity. There were no significant differences in the cyclic AMP levels in aortas of hypertensive and normotensive rats (9.3 ± 0.9 and 10.6 ± 1.1 pmoles/mg of protein, respectively; $N = 11$). The cyclic AMP-de-

TABLE 1

Ca^{++} uptake by aortic microsomal vesicles of 28–30-day-old spontaneously hypertensive rats and normotensive controls

Assay conditions are described under MATERIALS AND METHODS. The reaction was carried out in the presence of 10 μM $CaCl_2$ in buffer at 30° for 10 min. Values are means and standard errors for six rats.

Rats	Calcium uptake	
	−Cyclic AMP	1 μM cyclic AMP
	nmoles/mg/min	
Normotensive	1.64 ± 0.03	1.96 ± 0.01
Hypertensive	1.56 ± 0.02	1.70 ± 0.04[a]

[a] Significantly different from normotensive control ($p < 0.05$).

pendent protein kinase activity was determined in the 27,000 × *g* supernatant of aortic homogenates. A comparison of cyclic AMP-dependent and -independent protein kinase activities in aortas of both groups of animals is shown in Fig. 6. In hypertensive rats both cyclic AMP-dependent and -independent protein kinase activities were significantly reduced ($p < 0.05$), by 42% and 34%, respectively. In young animals (28–32 days) cyclic AMP-dependent and -independent protein kinase was reduced by 38% and 37%, respectively ($N = 4$).

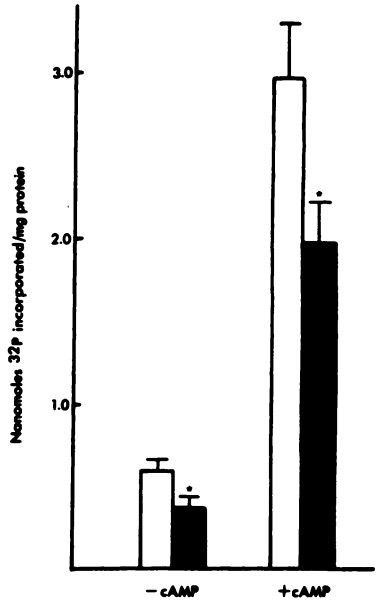

FIG. 6. *Cyclic AMP-dependent and -independent protein kinase activity in 27,000 × g supernatant of thoracic aortas of spontaneously hypertensive (solid) and normotensive (open bars) Kyoto Wistar rats*

Each value is the mean ± standard error of seven experiments. For each experiment the tissue extract was prepared from aortas of five to seven rats.

DISCUSSION

There are at least two major sources of Ca^{++} for activation of smooth muscle contraction: the extracellular space and intracellular storage sites (36–38). It is established that the magnitude of smooth muscle contraction is related to the availability of free cytoplasmic Ca^{++} through either Ca^{++} influx or release from subcellular storage sites (39). Consequently an alteration in Ca^{++} regulation has been postulated as a cause of defects in vascular smooth muscle observed in hypertension, including the supersensitivity of strips of arteries to excitatory agents such as potassium and norepinephrine (40, 41) and a decreased rate of relaxation (42). The current findings suggest an alteration in Ca^{++} regulation in vascular smooth muscle from spontaneously hypertensive and normotensive rats that involves both sources of Ca^{++}.

The total tissue Ca^{++} content of hypertensive aorta was the same as that of the normotensive rats at 31 days of age and slightly increased at 12–16 weeks of age. Similar results have been reported by other investigators (43, 44). Only after 1 year did a significant difference ($p < 0.05$) appear, with hypertensive tissue containing more calcium. Both the magnitude of the differences between the two groups and the tissue calcium content itself increase with age. The changes associated with age may be dependent on connective tissue content or may be related to the pathogenenis of hypertension. In spontaneously hypertensive rats blood pressure is markedly elevated by the age of 12 weeks; if the increase is related to smooth muscle tone, it is likely that the total tissue Ca^{++} may not reflect the small flux of ionized Ca^{++} presumed to be important in the contraction mechanism. The differences observed between Kyoto Wistar normotensive and Sprague-Dawley rats emphasize the importance of relevant controls for studies on spontaneously hypertensive rats.

In the calcium efflux experiments, it seems reasonable to consider the rapidly exchanging component as the extracellular space, and the slowly exchanging component as the intracellular space, as suggested by Jones and Karreman (31). When efflux occurs in calcium-free PSS, the slower rate of exchange can be explained by a lack of competition for the ^{45}Ca bound to the tissue and cell surfaces. However, the differences in the slow component observed in adult rats may suggest a "leakier" plasma membrane. Such leakiness, like the enhanced cellular ^{45}Ca uptake, might result in altered calcium distribution in the vascular smooth muscle cell in hypertensive animals. However, since this phenomenon was not observed prior to the onset of hypertension, it is probably a result of the elevation of blood pressure.

There is considerable evidence that microsomes (sarcoplasmic reticulum) serve as the storage site of Ca^{++}. Aspects of regulation of calcium transport by smooth muscle microsomes have been intensively studied (13–16). We have recently proposed that the phosphorylation of a microsomal protein component with an approximate molecular weight of 44,000 is responsible for the stimulation of Ca^{++} uptake mediated by cyclic AMP (41, 42). In view of these observations, we suggest that reversible phosphorylation of microsomal membranes may play an important role in the regulation of aortic microsomal calcium transport by cyclic AMP in vascular smooth muscle in a manner similar to that suggested for cardiac and skeletal muscle of spontaneously hypertensive rats.

The microsomal fraction of vascular smooth muscle of spontaneously hypertensive rats shows a reduction in calcium uptake ability compared with normotensive controls (9–11). However, Wei *et al.* (45) failed to observe differences between hypertensive and normotensive control rats in the Ca^{++} uptake ability of microsomes isolated from vascular smooth muscle. There appear to be at least two possible reasons for this discrepancy. Wei *et al.* utilized mesenteric arteries, whereas other investigators used aortas as the source of microsomes. Also, Wei *et al.* did not use Kyoto Wistar control rats. In the present study we observed reduced phosphorylation of aortic microsomal protein from the hypertensive rats (Fig. 4). Furthermore, Ca^{++} uptake by the microsomal fractions of hypertensive aorta was reduced compared with the normotensive control in the presence and absence of cyclic AMP (Fig. 5). Limas and Cohn (43) have recently reported a reduction in Ca^{++} uptake ability and a significant decrease in cyclic AMP-dependent phosphorylation of cardiac sarcoplasmic reticulum in hypertensive rats. Turnover of the phosphoryl moiety of phosphoproteins, which has been recognized as an important regulatory mechanism of cellular function in Ca^{++} transport in sarcoplasmic reticulum (12), seems to have been altered in the sarcoplasmic reticulum isolated from the cardiovascular tissues of hypertensive rats.

The time study on the pathogenesis of the disease process allowed separation of some prehypertensive events from hypertensive changes. Reduced Ca^{++} uptake by microsomes treated with cyclic AMP and decreased cyclic AMP-dependent protein kinase activity in tissue extracts preceded the development of elevated pressure. These observations suggest that reduced membrane phosphorylation and Ca^{++} uptake by the microsomal fraction of aorta from hypertensive animals may play a major role in the pathogenesis of hypertension.

Abnormalities in cyclic AMP metabolism that resulted in reduced levels have previously been related to intracellular regulation of vascular tone in spontaneously hypertensive rats (17–25). We observed no significant differences in the levels of cyclic AMP between hypertensive and normotensive rats. From the present data it seems likely that the reduced Ca^{++} uptake by aortic microsomes of hypertensive rats may be due to a less responsive effector system for cyclic AMP. Several other lines of evidence support the view that there is a defect in the intracellular reception of the cyclic AMP message in blood vessels of hypertensive animals. Cohen and Berkowitz (44) have recently shown that aortic strips from hypertensive rats show reduced relaxation compared with controls following treatment with dibutyryl cyclic AMP. Similarly,

Triner *et al.* (23) have observed that the relaxing effects of isoproterenol and theophylline are more severely reduced in hypertensive than in normotensive rats. Several explanations for such reduced relaxation in the hypertensive animals can be considered: (a) dibutyryl cyclic AMP enters the vascular smooth muscle cells of hypertensive rats at a significantly slower rate than in normotensive animals; (b) degradation of the nucleotide by phosphodiesterase is increased; (c) synthesis of the nucleotide is reduced; or (d) a decrease in the expression of the cyclic AMP message due to an inherent or induced defect in protein kinase activity has occurred. The current findings suggest that this impairment could be the result of any one (or more) of the altered mechanisms involved in the process of phosphorylation and dephosphorylation. Since our data are expressed per milligram of protein, one must be cautious in interpreting these results, because vessels from hypertensive animals contain more connective tissue and more rough endoplasmic reticulum than vessels from normal animals. Although the yield of microsomal pellet in hypertensive and normotensive rats was not different (9), the possibility of differential contamination by other subcellular components cannot be excluded.

Experiments to determine whether any of the subunits of protein kinase are altered in spontaneously hypertensive rats are in progress.

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