Possible Role of Phosphorylation-Dephosphorylation in the Regulation of Calcium Metabolism in Cardiovascular Tissues of SHR

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SUMMARY Spontaneously hypertensive rats (SHR) and Wistar-Kyoto normotensive rats (WKY) were compared for phosphorylation-dephosphorylation mechanism(s) in aorta, caudal artery, inferior vena cava, and right and left ventricles. Reduction of cAMP-induced phosphorylation of microsomes and cAMP-dependent protein kinase activity was significant in the aorta and caudal artery of SHR compared with WKY. These changes were not observed in the vena cava of SHR. Phosphoprotein phosphatase activity was significantly increased (p < 0.05) in the soluble fraction of arterial smooth muscle. No changes were observed, however, in the myocardium or vein. Furthermore, the extent of phosphorylation, and Ca\(^{2+}\) uptake ability and the protein kinase activity in the soluble and the microsomal fractions were not reduced in the myocardium of SHR compared with WKY. These data suggest that phosphorylation-dephosphorylation mechanisms are altered in the microsomal fraction of the aorta and caudal artery of SHR, which may result in reduced Ca\(^{2+}\) uptake by the intracellular organelle. The changes observed could have a significant effect on vasodilatation of arteries in the hypertensive state. The lesion appears specific to the arterial smooth muscle in the cardiovascular tissues. (Hypertension 2: 207-214, 1980)

KEY WORDS • spontaneous hypertension • calcium • heart and blood vessels • membrane phosphorylation

In considering possible explanations for increased peripheral resistance, which may be regarded as a primary characteristic of essential hypertension, the physiological state of vascular smooth muscle cell appears pivotal. Vascular smooth muscle strips from hypertensive rats show reduced relaxation compared with the normotensive control rats after treatment with dibutyryl cAMP or treatment with isoproterenol and theophylline in washout experiments following contractility induced by KCl. This defect in the relaxing ability of the vascular smooth muscle from hypertensive animals could lead to an increased vascular tonic resistance. An alteration in Ca\(^{2+}\) regulation has been postulated as a cause of increased vascular resistance and the rate of relaxation. The removal of Ca\(^{2+}\) from the cytoplasm and consequent initiation of relaxation process in smooth muscle are probably accomplished by energy-dependent calcium transport in the intracellular organelles and extrusion across plasma membrane.

Insight into the molecular mechanisms involved in the postulated role of cAMP-dependent protein kinase in Ca\(^{2+}\) transport in the sarcoplasmatic reticulum (SR) comes from the studies of Hicks, Shigekawa, and Katz. The phosphorylation of the 22,000 dalton protein phospholamban of cardiac SR by cAMP-dependent protein kinase is shown to accelerate Ca\(^{2+}\) sequestration. Furthermore, the extent of phosphorylation corresponds closely with the increased rate of Ca\(^{2+}\) transport. We have recently reported some aspects of regulation of Ca\(^{2+}\) transport by rat aortic microsomes. We found that cAMP-dependent protein kinase augmented phosphorylation of microsomal protein, and phosphorylated microsomes exhibited enhanced Ca\(^{2+}\) uptake. These data suggest a modulatory role for cAMP-dependent protein kinase in Ca\(^{2+}\) transport in the vascular smooth muscle, and hence in the contraction-relaxation process.

The microsomal fraction of the vascular smooth muscle of spontaneously hypertensive rats (SHR)
showed a reduction in $\text{Ca}^{2+}$ uptake ability compared with normotensive controls. A concomitant reduction in microsomal membrane phosphorylation has been observed in the cardiovascular tissues of SHR. The molecular basis for altered $\text{Ca}^{2+}$ regulation in hypertensive animals could therefore be due to a defect in phosphorylation-dephosphorylation mechanisms. Present studies were undertaken to answer the following questions: 1) Is the decreased phosphorylation of microsomal membranes due to an increased activity of phosphoprotein phosphatase or to reduced activity of cAMP-dependent protein kinase? 2) Are the alterations in the activities of protein kinase, phosphoprotein phosphatases, and membrane phosphorylation specific to the systemic arteries and left ventricles, which are subjected to high "stressed wall" pressure, or do they generally appear in other cardiovascular tissues?

**Materials and Methods**

Adult male Wistar-Kyoto spontaneously hypertensive rats (SHR), and Wistar-Kyoto normotensive rats (WKY) 12-16 weeks old, were used. The SHR maintained at the University of Iowa are inbred descendants of the hypertensive Wistar strain developed by Okamoto and Aoki. The control rats were raised under conditions identical to those used for the hypertensive animals. Preoperative systolic blood pressures were determined in the unanesthetized state by the tail plethysmographic method, with an automated cuff inflator pulse-reading system manufactured by Technilab Instruments. The $\text{Ca}^{2+}$ uptake was measured in 0.12 ml containing 40 mM acetate (pH 6.0), 18 mM NaF, 3.7 mM theophylline, 0.25 mg histone, 18 mM MgCl$_2$, 0.1 mM [γ-32P] ATP (2-6 × 10$^6$ cpm), and 5 µM cAMP when added. The reaction was initiated by addition of 80-100 µg of protein from various tissue fractions and incubated at 30°C for 10 minutes. Reaction was terminated by addition of 2.0 ml 10% ice-cold trichloroacetic acid (TCA), and filtered through Millipore filters and washed three times with 5.0 ml 10% ice-cold TCA.

**Phosphorylation of Microsomal Protein**

Microsomal vesicles were phosphorylated in 200 µl of a solution containing 0.05 M Tris HCl, pH 7.4, 18 mM NaF, 22 mM MgCl$_2$, 0.1 mM [γ-32P] ATP (3-5 × 10$^6$ cpm), and 150-200 µg of microsomal protein. The reaction was initiated by the addition of phosphatases and incubated at 30°C for 10 minutes. The reaction was terminated by the addition of 10% ice-cold TCA and filtered through Millipore filters.

**Measurement of Phosphoprotein Phosphatase**

For phosphoprotein phosphatase assay, 32P-histone (type II A) or 32P-protamine were used as substrates. The 32P-labelled substrates were prepared according to the methods of Meisler and Langan and Maeno and Greengard. Briefly, the method consists of incubating histone and protamine with [γ-32P] ATP in the presence of cAMP-dependent protein kinase. One ml of the incubation mixture contained 46 µg protein kinase; 50 µmol sodium acetate buffer, pH 6.4; 1 mg protamine or histone; 0.2 µmol of ATP (0.1 µmol ATP for protamine), γ-32P-ATP (5.10 × 10$^6$ cpm); 10 µmol of magnesium acetate; 10 µmol of sodium fluoride; 2.0 µmol of theophylline; 0.3 µmol of ethylene glycol bis (β-amino ethylene) N-tetracetic acid; and 5.0 nmo! of cAMP. The mixture was incubated at 37°C for 45 minutes and the reaction was terminated by adding 0.25 ml of 100% TCA. The
resulting precipitate was centrifuged, washed two times by suspending it in water and reprecipitating with 20% TCA, and then dialyzed against distilled water. The amount of phosphate incorporated was calculated from the \( ^{32}P \)-phosphate incorporated. The \( ^{32}P \)-histone contained 25 nmol of \( ^{32}P \)/mg histone, and \( ^{32}P \) protamine contained 8 nmol of \( ^{32}P \)/mg protamine. For the measurement of phosphoprotein phosphatase activity, the reaction mixture in 0.15 ml contained 50 mM MgCl\(_2\), 1 mM dithiothreitol, 100 \( \mu \)g \( ^{32}P \)-labeled substrate, and 20-40 \( \mu \)g tissue fraction. The incubation was carried out at 30°C for 10 minutes, and the reaction was terminated by adding 0.4 ml of 25% TCA solution. After centrifugation, 0.4 ml of supernatant was added into tubes containing 50 \( \mu \)l of 100 mM KH\(_2\)PO\(_4\), and 150 \( \mu \)l of 5% ammonium molybdate. The phosphomolybdate complex was extracted with 1.0 ml of isobutanol, and the radioactivity in 0.5 ml of isobutanol was counted.

**Results**

The average blood pressure of SHR was 170 ± 8 mm Hg as compared with 140 ± 6 mm Hg for WKY. Biochemical characterization of the microsomal fraction, as reported earlier\(^a\), for the vascular smooth muscle, was carried out by the determination of cytochrome oxidase activity. The activities per milligram of protein were less than 8% of that in the mitochondrial fraction and there were no differences between SHR and WKY. Electron micrographs of the microsomal preparation from myocardium showed that this fraction consisted of smooth membrane vesicular structures and was devoid of contractile proteins. No intact mitochondrial fragments could be identified in this preparation. There was no significant difference in the yield of microsomal protein for the different rats. Determinations of microsomal Ca\(^{2+}\) uptake and binding and protein kinase activities were carried out under conditions of linearity with respect to time of incubation and protein concentration.

**Calcium Binding and Uptake**

We have shown earlier\(^a\), \(^b\) that calcium uptake by microsomal vesicles isolated from aortae of hypertensive rats was significantly reduced \((p < 0.05)\) compared to normotensive controls. In contrast, Ca\(^{2+}\) binding (fig. 1A) and Ca\(^{2+}\) uptake (fig. 1B) in the sarcoplasmic reticulum (SR) isolated from right and left ventricles of hypertensive rats were not changed in SHR compared with the normotensive controls. Similar results were obtained when Ca\(^{2+}\) uptake was studied in the SR phosphorylated in the presence of 5 \( \mu \)M cAMP or 5 \( \mu \)M cAMP and 0.1 mg/ml cAMP-dependent protein kinase. Comparison of the Ca\(^{2+}\) binding and uptake ability between left and right ventricles of the same type of animal showed consistently higher values for the left ventricles than for the right ventricles.

**Figure 1.** Calcium binding (A) and calcium uptake (B) in the sarcoplasmic reticulum (SR) isolated from right and left ventricular walls of spontaneously hypertensive rats (shaded bars) and Wistar-Kyoto normotensive rats (open bars) in the presence and absence of cAMP and cAMP plus cAMP-dependent protein kinase. Each value is the mean ± SE of five experiments. For each experiment SR were prepared from the myocardium of four to five rats.
cAMP-Stimulated Phosphorylation of Microsomes

The cAMP-dependent and -independent phosphorylation of SR isolated from right and left ventricles is shown in fig. 4. In the SR isolated from the right ventricle, there were no differences between hypertensive and normotensive rats either in the extent of stimulation achieved over the basal value by 1 μM cAMP or in the levels of phosphorylation. In the SR of the left ventricle, however, P incorporation in the absence and presence of 5 μM cAMP was significantly higher in the hypertensive rat as compared to the normotensive rat. The net stimulation by cAMP was 15%-20% over the basal value.

Phosphoprotein Phosphatase Activity

We compared phosphoprotein phosphatase activity of microsomes and soluble fraction isolated from vascular smooth muscle and also in the right and left ventricles from both the groups. The enzyme activity was consistently increased in the soluble fraction of the aorta and caudal artery of SHR compared with WKY, with significant differences (p < 0.05) observed in the caudal artery (fig. 5A). No changes were observed, however, in the inferior vena cavae (fig. 5A), or in the right and left ventricles (fig. 5B). Addition of 1 mg/ml bovine heart protein kinase to the phosphoprotein assay mixture did not alter the observed differences (data not given). Phosphoprotein phosphatase activity in the soluble fraction of caudal artery was found to be linear in the range of 2–20 minutes and 5–100 μg protein. At all incubation intervals tested, and at protein concentrations from 10 μg and higher, the phosphatase activity was consistently
increased in hypertensive rats compared with normotensive controls (fig. 6). The increase in phosphorylation-dephosphorylation activity in the caudal artery of SHR was due to an increase in \( V_{\text{max}} \) rather than \( K_m \). The \( V_{\text{max}} \) values for phosphohistone and phosphoprotamine were doubled in SHR compared with WKY (table 1) with no change in apparent \( K_m \). The enzyme activity was determined in the supernatant and the sarcoplasmic reticulum preparations in the presence of several divalent cations. Divalent cations \( Mn^{2+} \), \( Mg^{2+} \), and \( Ca^{2+} \) stimulated the phosphatase activity in the vascular smooth muscle and in cardiac muscle (data not given). In all the preparations tested, \( Mn^{2+} \) was the most potent stimulator of the divalent cations. The enzyme activity was significantly higher in the supernatant fraction of the caudal artery of SHR as compared with WKY with all the divalent cations tested; however, no differences were observed in the vena cava and myocardium between the two groups.

Discussion

A defect has been reported in the relaxing ability of the vascular smooth muscle from hypertensive animals after treatment with dibutyryl cyclic AMP and isoproterenol. Physiological and pharmacological regulation of contraction or relaxation of vascular smooth muscle is determined by the concentration of activator Ca\(^{2+}\) in the sarcoplasm. The removal of Ca\(^{2+}\) from the cytoplasm and consequent initiation of relaxation in smooth muscle involves two important systems: 1) sequestration into intracellular structures, including both mitochondria and sarcoplasmic reticulum; and 2) efflux across the plasma membrane. Abnormalities in each of these mechanisms have been implicated in the pathogenesis of hypertensive disorders.

From studies on microsomal fraction of smooth muscle, Ford and Hess\(^{27}\) concluded that the microsomes sequestered sufficient Ca\(^{2+}\) and that this fraction has the capability to be both sink and source for the activator Ca\(^{2+}\) in the sarcoplasm. The removal of Ca\(^{2+}\) from the cytoplasm and consequent initiation of relaxation in smooth muscle involves two important systems: 1) sequestration into intracellular structures, including both mitochondria and sarcoplasmic reticulum; and 2) efflux across the plasma membrane. Abnormalities in each of these mechanisms have been implicated in the pathogenesis of hypertension.\(^{36-37}\)

From studies on microsomal fraction of smooth muscle, Ford and Hess\(^{28}\) concluded that the microsomes sequestered sufficient Ca\(^{2+}\) and that this fraction has the capability to be both sink and source for the activator Ca\(^{2+}\) in the sarcoplasm. Observations made in this laboratory\(^{19}\) and by others\(^{22-27}\) demonstrate that incubation of smooth muscle microsomal fraction with cyclic AMP-dependent protein kinase enhances energy-dependent Ca\(^{2+}\) sequestration, which suggests a modulatory role for cyclic AMP-dependent protein kinase in vascular smooth muscle. Previous observations of reduced Ca\(^{2+}\) uptake by aortic microsomes of SHR compared with WKY\(^{18-19}\) led us to investigate further the biochemical basis for this defect. Our results indicate that cyclic AMP-dependent protein kinase activity in the microsomal and soluble fractions of the caudal artery and aorta of SHR was significantly reduced compared with those of WKY. The stimulation of protein kinase activity by cyclic AMP was also reduced in SHR. This could result in a decrease in the expression of cyclic AMP message due to an inherent or induced defect in cyclic AMP-dependent protein kinase mediated membrane phosphorylation.
Another mechanism that could influence the net membrane phosphorylation and Ca\(^{2+}\) metabolism is a change in the dephosphorylation process, probably due to the altered activity of phosphoprotein phosphatase. Dephosphorylation of phosphorylated cardiac sarcoplasmic reticulum has been shown to be catalyzed by both membrane associated\(^{10-12}\) and soluble\(^{10}\) phosphoprotein phosphatase. It was, further, demonstrated that dephosphorylation of the 22,000 dalton phosphoprotein of cardiac sarcoplasmic reticulum catalyzed by an intrinsic phosphoprotein phosphatase was associated with a decrease in the rate of Ca\(^{2+}\) transport by these membranes.\(^{10}\) It has been suggested that similar mechanisms may be regulating Ca\(^{2+}\) metabolism in vascular smooth muscle.\(^{10}\) Our results indicated an increase in the phosphoprotein phosphatase activity in the soluble fraction of arterial smooth muscle of SHR compared with that of WKY (figs. 5 and 6). The increase in activity was associated with a change in \(V_{\text{max}}\). The combined effect of these changes, i.e., a decrease in protein kinase activity and an increase in phosphoprotein phosphatase activity, could result in a reduced membrane phosphorylation and Ca\(^{2+}\) uptake by membrane vesicles in the arterial smooth muscle cell.

Recently, Kuo et al.\(^{11}\) reported the role of "wall stress" of blood vessels on the levels of cGMP- and cAMP-dependent protein kinase(s). To test the effect of intravascular pressure on cAMP-dependent and -independent protein kinases, comparisons were also made between inferior vena cava of SHR and WKY. Since we did not find differences in the cAMP-dependent and -independent protein kinases and phosphoprotein phosphatase activity in the inferior vena cava, it is suggested that the lesion is specific to the arterial smooth muscle. Furthermore, in the left and right ventricles we observed no differences between SHR and WKY for Ca\(^{2+}\) uptake by SR, cAMP-dependent and -independent protein kinase, and phosphoprotein phosphatase activity in soluble and microsomal fractions. Limas and Cohn,\(^{12}\) however, have reported reduced Ca\(^{2+}\) uptake and a

### Table 1. Kinetic Constants for Phosphoprotein Phosphatase in Soluble Fraction of Caudal Artery*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>WKY</th>
<th>SHR</th>
<th>WKY</th>
<th>SHR</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(K_m) mg/ml</td>
<td>(K_m) (\mu)M [(^{32})P]</td>
<td>(K_m) mg/ml</td>
<td>(K_m) (\mu)M [(^{32})P]</td>
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<tr>
<td>Histone</td>
<td>0.65</td>
<td>25</td>
<td>0.65</td>
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<tr>
<td>Protamine</td>
<td>0.36</td>
<td>2.2</td>
<td>0.38</td>
<td>2.3</td>
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*Each value is the mean of two determinations. \(\mu\)M [\(^{32}\)P] refers to molarity of bound \(^{32}\)P. \(V_{\text{max}}\) is expressed as nM \(^{32}\)P released per mg protein/min at 30°. WKY = Wistar-Kyoto normotensive rat; SHR = spontaneously hypertensive rat.
decrease in membrane phosphorylation and protein kinase activity in the SR isolated from the myocardium of SHR compared with WKY. The reason for this discrepancy is hard to explain. There appear to be at least two possible reasons: 1) source of hypertensive and control animals; and 2) ventricular wall employed in the present study compared with the whole heart. Biochemical observations made in the present investigation correlate well with the hemodynamic responses and myocardial function in SHR. Pfeffer and Frohlich\textsuperscript{9} have shown that cardiac output in 9- to 12-week SHR was about 22% above that of age-matched normotensive controls. Similar observations have been made for other experimental models of hypertension.\textsuperscript{9,44} Our data of increased SR membrane phosphorylation and protein kinase activity in the left ventricle of SHR compared with WKY would provide a basis for observed increased cardiac output and myocardial function in the hypertensive state.

These data suggest that alteration in phosphorylation-dephosphorylation mechanisms of SR of arterial smooth muscle has occurred in SHR. Further, these changes are specific for arterial smooth muscle among the cardiovascular tissues. In smooth muscle, increased intracellular levels of cAMP inhibit tension development. This effect has been attributed to enhanced Ca\textsuperscript{2+} binding by membrane fractions, thereby lowering intracellular free Ca\textsuperscript{2+}. The data presented here are suggestive of changes that could influence calcium metabolism in the vascular smooth muscle in such a manner that it could result in an increase in free Ca\textsuperscript{2+} levels in vascular smooth muscle in the hypertensive state. These results, however, should be taken with caution because they do not unequivocally demonstrate a reduced Ca\textsuperscript{2+} sequestering ability, with the implication of higher cytoplasmic [Ca\textsuperscript{2+}] and increased tone in the hypertensive animals.

Another possible mechanism that could imply a direct role of the changes observed in protein kinase activity in regulating smooth muscle tone comes from the observations of Adelstein et al. They have demonstrated that a cAMP-dependent protein kinase in smooth muscle can phosphorylate the myosin light chain kinase. This decreases the activity of the myosin light chain kinase, and therefore the degree of myosin phosphorylation that would decrease Ca\textsuperscript{2+} stimulated interaction between actin and myosin. Thus it is possible that the decreased cAMP-dependent protein kinase activity in the vascular smooth muscle of hypertensive animals might be associated with a more active myosin light chain kinase, more phosphorylated myosin, and a higher level of tone. It will be of interest to learn whether these enzymes are altered in the hypertensive state.

References

11. Tada M, Kircherger MA, Katz AM: Phosphorylation of a 22,000 dalton component of the cardiac sarcoplasmic reticulum by adenosine 3\textquotesingle,5\textquotesingle-monophosphate-dependent protein kinase. J Biol Chem 250: 2640, 1975

27. Friedman SM: An ion exchange approach to the problem of intracellular sodium in the hypertensive process. Circ Res 34-35 (suppl 1) 1-123, 1974


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R C Bhalla, R V Sharma and S Ramanathan

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