Abnormality of Calmodulin Activity in Hypertension
Evidence of the Presence of an Activator

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Abstract
An apparent increase of calmodulin (CaM) activity was previously observed in the heart and kidney but not in the liver of spontaneously-hypertensive rats (SHR) and mice compared with their corresponding normotensive controls. As this change was due to an elevated recovery of CaM in the organs of the hypertensive animals, the present study was designed to evaluate its activity in hypertension. A CaM activator, detected in heart and kidney supernatants from hypertensive animals, was found to be responsible for this enhanced recovery. Similar results were obtained with passaged, cultured aortic smooth muscle cells from SHR, indicating that the anomaly was not a mere consequence of elevated blood pressure but rather a genetic expression of cells of hypertensive origin. The activator was heat stable, nondialyzable, and recovered in the fraction precipitated with 30–50% ammonium sulfate. Preliminary extraction studies suggest that the activator is contained in a glycolipid fraction. The stimulation of phosphodiesterase by this activator was calcium and CaM dependent. The activator appears to affect the affinity of the phosphodiesterase for CaM rather than the maximal stimulation. The activator was also present at a low concentration in the heart and kidney of normotensive animals. These findings indicate that at least some of the calcium abnormalities implicated in the pathogenesis of hypertension could be the result of interactions between CaM, calcium, and this activator.

Introduction
Abnormalities of cellular calcium transport, binding, and sequestration, which may lead to alterations in free cytosolic calcium levels, have been noted in hypertension (1–6). Arterial smooth muscle cell tension and therefore peripheral vascular resistance appear to be controlled by variations in free cytosolic calcium levels (7). Many studies have uncovered several anomalies of key Ca2+-dependent enzymes and cellular processes in hypertension including Ca2+-Mg2+ ATPase (2, 8), calmodulin (CaM)1-dependent ATPase (9), and phospholipase A2 (10). The cellular hyperplasia seen in hypertension may also be related to changes in intracellular Ca2+ (11–13). CaM is known to regulate smooth muscle tone by activation of myosin light-chain kinase or by binding caldesmon, a contractile inhibitory protein, resulting in actin-myosin interactions (14–16). It therefore appears logical to postulate that Ca2+-dependent enzyme alterations could play an important role in hypertension.

CaM is a major intracellular Ca2+ receptor that can control the activity of many key enzymes and cellular processes, including those just mentioned. In vitro, CaM has been shown to suppress differences in calcium binding and ATP-dependent accumulation between spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto rats (WKY) (17). Its role in hypertension is, however, still not clear.

CaM content can be determined by direct RIA or by a radioenzymatic assay based on the activation of CaM-deficient cyclic nucleotide phosphodiesterase (PDE) (18). Using the latter assay, we have observed an apparent increase of CaM content in the heart and kidney but not in the liver of spontaneously hypertensive mice (SHM) (Schlager strain) and rats compared with their corresponding normotensive counterparts (19, 20). However, with a direct RIA, Higaki et al. (21) reported a decrease of CaM levels in SHR organs compared with those from normotensive WKY.

In this study, we report the presence of an activator of CaM in the heart, kidney, and in cultured aortic smooth muscle cells from hypertensive animals with an apparent increase of activity in organs of hypertensive origin.

Methods
Animals. These experiments were performed on 10-wk-old SHM, SHR, and age-matched, normotensive, randomly bred mice (NM) and rats (WKY).

Preparation of tissue supernatants. The mice and the rats were killed by exsanguination under anesthesia. Their hearts and kidneys were quickly removed and frozen in liquid nitrogen. Each frozen organ was weighed and pulverized with a caulk Vari-Mix II-M for 10–20 s. The powders obtained were resuspended in 5 vol of 10 mM N-tris(hydroxymethyl) methyl-2-amino ethanesulfonic acid (TES) buffer (pH 7) containing 1 mM DTT, 1 mM EGTA, and 50 μg/ml leupeptin. These homogenates were heated at 90–100°C for 3 min, then cooled rapidly to 4°C. They were sonicated at 12 μm for 10 s and centrifuged at 37,000 g for 30 min. The supernatants were stored at −70°C until they were analyzed.

Aortic smooth muscle cells were obtained from 10-wk-old male SHR and WKY by the "explant" technique (22, 23). The cells were maintained in DMEM supplemented with 10% bovine serum (pretested for its ability to support vascular smooth muscle cells in culture), 8 mM of Hepes buffer, 100 U/ml of penicillin and 100 μg/ml of streptomycin. These cells present a hill and valley morphology after confluence, and are positively stained with antimyosin (smooth muscle) antibodies (Biomedical Technologies, Inc., Cambridge, MA). The experiments were performed with cells after the 11th passage.

Precipitation of supernatant protein with ammonium sulfate. Pooled supernatants of kidneys from both hypertensive and normo-
tensive mice were precipitated with ammonium sulfate into five fractions. Fraction 1 represented precipitated proteins obtained with 0–30% saturation of ammonium sulfate after centrifugation at 15,000 g; fraction 2, 30–50%; fraction 3, 50–80%; and fraction 4, 80–100%. Fraction 5 was derived after 100% saturation of ammonium sulfate. Each fraction was redissolved in 10 mM TES buffer containing 1 mM DTT and dialyzed against the same buffer.

Preparation of ether extract. 50 μl ethyl ether was added to a portion of fraction 2 and fraction 5 (100 μl). The mixture was agitated vigorously and separated into two layers. The ether was then evaporated under a stream of nitrogen, TES-DTT buffer was added to the residue, and the mixture was sonicated. The resultant product is referred to as the ether extract.

Measurement of CaM: activation of CaM-dependent PDE. The measurement of cGMP-PDE activity is based on the conversion of [3H]GMP into [3H]GMP and its subsequent hydrolysis by snake venom into [3H]guanosine (24, 25). The samples (tissue supernatant, aortic smooth muscle cells, ether extract, or lipid fraction) were added to a mixture containing 60 mM TES, 3 mM MgCl₂, 0.8 mg/ml BSA, 25 μM EGTA, 50 μM CaCl₂, 1 mM DTT, 0.5 μM cGMP and [3H]-cGMP (~10–15×10³ cpm) at a final pH of 7.5 for a total volume of 200 μl. The reaction was started by the addition of a fixed quantity of CaM-deficient PDE (Sigma Chemical Co., St. Louis, MO). Incubation, carried out for 30 min at 30°C, was terminated with 25 μl of a medium containing 50 mM EDTA, 5 mM 1-methyl-3-isobutylxanthine, 15 mM cGMP, and 15 mM 5GMP. The second reaction was initiated by the addition of 25 μl (10 mg/ml) of snake venom (Crotalus atrox). After 10 min, the incubation was stopped with 750 μl of a second terminating medium containing 0.1 mM guanosine. The total incubation mixture was then applied onto a column of QAE-Sephadex A-25 (formate form) to separate labeled nucleotides from the labeled nucleosides. The resin was eluted with 3.5 ml of 20 mM ammonium formate (pH 6.5), and the radioactivity of the eluate containing [3H]guanosine was determined in a liquid scintillation counter. A standard curve of PDE stimulation by CaM was obtained by the addition of different concentrations of purified bovine brain CaM (Calbiochem-Behring Diagnostics, La Jolla, CA) (0.1–10 ng/tube) to the reaction mixture in the presence of 25 μM EGTA and 50 μM CaCl₂. For the evaluation of CaM recoveries, the samples were also assayed in the presence of known levels of purified CaM.

Measurement of protein content. Proteins were measured by the method of Lowry et al. (26) using BSA as a standard.

Statistical analysis. Statistical analyses were performed with the Student’s t test. The values are expressed as means±SE.

Results

Apparent CaM content in the hypertensive heart and kidney. A comparison of the CaM content of hypertensive and normotensive kidneys demonstrated no significant difference between the SHR and WKY groups when the supernatants were highly diluted (600 times), but a significant difference was evident when CaM was measured in supernatants which were 10-fold (60 times) more concentrated (Fig. 1). In these concentrated samples, the apparent CaM content was higher in kidney supernatants from hypertensive rats compared with the corresponding normotensive controls. A similar difference was observed between the NM and SHM strains (data not shown).

Since the latter species represents a better model for the study of the genetics of hypertension, further experiments were performed on this species. Fig. 2 shows that the elevation of apparent CaM content was linear with protein concentration in the NM heart supernatant but was nonlinear in the case of the SHM.

Endogenous content and recovery of CaM in hypertensive supernatants. These results led us to evaluate the recovery of exogenously added CaM, which is defined as the apparent (measured) CaM content after the addition of a fixed quantity of standard CaM, minus the apparent CaM content before the addition of standard CaM, divided by the amount of added standard CaM. Recovery = CaMapp (with added CaM) − CaMapp (without added CaM)/CaMstandard, CaMapp = (CaMendo + CaMstandard) recovery. Using two different concentrations of standard CaM, it is possible to calculate, with the following formulae, the CaM recovery as well as the endogenous CaM content in tissues: CaMapp 1 = (CaMendo + CaMstandard 1) recovery, CaMapp 2 = (CaMendo + CaMstandard 2) recovery, Recovery = CaMapp 1 − CaMapp 2/CaMstandard 1 − CaMstandard 2, CaMendo = CaMapp 1 − (CaMstandard 1 + Recovery)/recovery. CaM recovery was significantly higher (P < 0.05) in hypertensive tissue supernatants than in the corresponding normotensive samples (Table I). This parameter was not dependent on the protein concentration in normotensive tissues but was in hypertensives, that is, CaM recovery increased linearly with the protein concentration in hypertensive supernatants (Fig. 3). The addition of escalating amounts of supernatant, which included both the activator and endogenous CaM, resulted in an increasingly greater apparent CaM content (Fig. 4). These results indicate the existence of an activator in hypertensive supernatants that directly or indirectly potentiated the stimulation of PDE by CaM. These considerations permit the direct estimation of this activator, based on the relationship between CaM recovery and tissue concentrations as shown in Fig. 3. Using the above mentioned formulae, we calculated the endogenous CaM content of the SHM and NM heart (Table II), and found it to be significantly lower in hypertensive than in normoten- sive supernatants. Similar results were obtained for kidney supernatants (data not shown).
Table I. CaM Recovery in Hypertensive and Normotensive Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Animals</th>
<th>CaM recovery*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>SHR (8)</td>
<td>311±59</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>WKY (8)</td>
<td>118±42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHM (8)</td>
<td>188±26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>NM (8)</td>
<td>111±18</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>SHM (10)</td>
<td>169±18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>NM (10)</td>
<td>102±14</td>
<td></td>
</tr>
</tbody>
</table>

* Mean±SE. Figures in parentheses indicate the number of animals used.

CaM content of cultured aortic smooth muscle cells. Apparent CaM content and CaM recovery were determined in aortic smooth muscle cells after the 11th passage. Similar to such organs as the heart and kidney, cultured aortic smooth muscle cells derived from SHR possessed the endogenous activator. The apparent CaM content increased linearly with the protein concentration in supernatants from aortic smooth muscle cells derived from WKY. On the other hand, supernatants derived from aortic smooth muscle cells from SHR showed increasingly greater apparent CaM content with increasing protein concentration. This nonlinear behavior can be explained again by higher recovery of CaM; 100% recovery was observed in cells from WKY, while 150% recovery was obtained with 20 ng and 400% with 100 ng of supernatant proteins from aortic smooth muscle cells from SHR.

Effect of supernatants from hypertensive and normotensive tissues on PDE activation curve by CaM. Fig. 5 shows that the hypertensive but not the normotensive sample modifies the standard curve of phosphodiesterase stimulation by CaM. At suboptimal concentrations of CaM, the hypertensive sample increased the PDE stimulation whereas it did not increase the maximal stimulation. The reciprocal transformation of these curves (Fig. 5, inset) demonstrated that the hypertensive sample decreases the concentration of CaM (from 1.1 to 0.4 ng) needed to reach 50% of the maximal stimulation of PDE. These results suggest that the activator present in hypertensive sample acts mainly by increasing the affinity of the PDE for CaM with no important effect on the maximal stimulation (from 2.9 to 2.6-fold stimulation).

Table II. Corrected CaM Content of Heart Supernatants from Hypertensive and Normotensive Mice

<table>
<thead>
<tr>
<th>Animals</th>
<th>&quot;Apparent&quot; CaM content</th>
<th>Recovery</th>
<th>&quot;Corrected&quot; CaM content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg · mg protein⁻¹</td>
<td>%</td>
<td>µg · mg protein⁻¹</td>
</tr>
<tr>
<td>SHM</td>
<td>1.17±0.12</td>
<td>158±20</td>
<td>0.57±0.041</td>
</tr>
<tr>
<td>NM</td>
<td>0.96±0.12</td>
<td>109±12</td>
<td>0.728±0.075</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data are mean±SE of 10 animals in each group.

Partial characterization of the activator. Table III enumerates the results of tissue supernatant fractionation by ammonium sulfate, which was performed in SHM and NM kidneys, as described in Methods. CaM was precipitated with 50–80% saturation of ammonium sulfate corresponding to fraction 3 (500-fold dilution) of hypertensive and normotensive tissues. The activator was found in fractions 2 (pellet and floating precipitate) and 5 after dialysis against TES-DTT buffer, as indicated by increased CaM recovery. It was significantly more active in fraction 2 of the hypertensive than in the normotensive kidney. The presence of the activator in fraction 5 of the two strains was detectable only in undiluted samples compared with fraction 2, which necessitated a 50-fold dilution (Table III).

Fractions 2 and 5 from SHM and NM revealed a displacement to the left of the standard curve of PDE stimulation by CaM and confirmed the greater effect of fractions from hypertensives (data not shown).

Fractions containing the activator could be heated at 100°C for 15 min with no effect on the CaM recovery.

Fraction 2 from SHM, which appeared to contain the highest levels of the activator, was further extracted with diethyl ether. Both the original fraction and its ether extract from hypertensives potentiated CaM stimulation of PDE, whereas the residual water phase showed no such effect (Fig. 6). A similar observation was made with fraction 5 (data not shown). Using ammonium sulfate precipitation followed by ether extraction, we can obtain an enriched preparation of the activator deprived of endogenous CaM. This component seems to possess a lipidic character.

To further narrow down the identity of the endogenous activator of CaM, the total lipids were extracted (hexane/isopropanol; 3:2) either from whole SHM heart homogenate or from supernatant, and fractionated by silicic acid column chromatography into four fractions (neutral lipids and FFA,
Figure 5. Effect of supernatants from normotensive and hypertensive heart on the activation curve of PDE by CaM. ○, standard curve; ▲, standard curve plus normotensive sample; ◼, standard curve plus hypertensive sample. The protein concentrations of normotensive and hypertensive samples were 110 ng/10 μl and 94 ng/10 μl, respectively. These experiments were performed in the presence of 25 μM EGTA and 50 μM CaCl₂. CaM₃ represents the added standard CaM plus endogenous CaM. Endogenous CaM was calculated as previously described. The reciprocal transformation of the data is shown in the inset. Data are means±SE of triplicate determinations.

PGs, glycolipids, phospholipids). The effect of these purified lipid fractions (devoid of endogenous CaM) on the CaM stimulation of PDE was evaluated as described in Methods. In the whole homogenate, the phospholipid- and glycolipid-containing fractions were active whereas in the supernatant, the glycolipid-containing fraction showed the most significant activation of CaM in the assay of PDE stimulation. The potential of PDE stimulation by the glycolipid-containing fraction from supernatant was observed to be calcium and CaM dependent. Studies to identify the structure of the glycolipid or of the chromatographically identical lipid activator of CaM are presently being performed in our laboratory.

Table III. Protein Fractionation of Kidney Supernatants from Normotensive and Hypertensive Mice

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration of ammonium sulfate (μg)</th>
<th>CaM content (μg·mg protein⁻¹)</th>
<th>Recovery (%)</th>
<th>Dilution fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertensive</td>
<td>0–30</td>
<td>5</td>
<td>88</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>30–50</td>
<td>12</td>
<td>155</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>50–80</td>
<td>19</td>
<td>83</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>80–100</td>
<td>6</td>
<td>81</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>Supernatant</td>
<td>2</td>
<td>218</td>
<td>1</td>
</tr>
<tr>
<td>Normotensive</td>
<td>0–30</td>
<td>2</td>
<td>84</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>30–50</td>
<td>6</td>
<td>110</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>50–80</td>
<td>25</td>
<td>99</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>80–100</td>
<td>10</td>
<td>93</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>Supernatant</td>
<td>1</td>
<td>154</td>
<td>1</td>
</tr>
</tbody>
</table>

Fractions were prepared as described in Methods.

CaM and calcium requirements for PDE stimulation by the partially purified activator (ether extract). Fig. 7 shows the requirement of CaM and Ca²⁺ for PDE stimulation by enriched fractions of the activator (ether extract). Basal PDE activity (100%) was determined in the absence of exogenous Ca²⁺ and EGTA. The addition of EGTA slightly inhibited PDE due to contamination by trace amounts of Ca²⁺ and CaM. The presence of either exogenous Ca²⁺ or EGTA only had a mild effect on PDE activity. The activator alone (ether extract) also had only a slight impact on the stimulation of PDE, which was increased considerably by the combination of CaM and the activator in the absence or presence of added calcium. The removal of contaminating calcium (which was high enough to allow a half maximal effect of CaM) by EGTA abolished the effect of the activator as well as that of CaM. Thus, the influence of the activator on PDE stimulation is dependent on the presence of both CaM and Ca²⁺.

Discussion

As mentioned before, the major intracellular Ca²⁺ receptor, CaM, regulates the activity of many enzymes and cellular processes including those altered in hypertension. Changes in calcium metabolism in hypertension have been studied by many groups. The cytosolic Ca²⁺ concentration, for example, has been found to be higher in hypertensives than in normotensives (4–6, 27, 28). However, most of the investigations into abnormal enzyme function in hypertension were performed in broken cell systems where exogenous Ca²⁺ concentrations were added. Thus, the differences observed between hyperton-
sives and normotensives could not be due to differences in intracellular Ca\(^{2+}\) concentrations, which also applies to the present study.

CaM content was reported to be unchanged or lower in hypertensive tissues (10, 21, 29). Our initial results (19, 20), however, showed an increase of CaM activity (in contrast to its content) in the heart and kidney of SHR and SHM. In this report, we demonstrated that this is due to the presence of an activator of CaM in the heart and kidney of SHR and SHM. In this study, we present data that the activator increases the concentration of CaM, and, consequently, CaM-dependent PDE activity. The stimulation of CaM-dependent PDE by the activator increases with the concentration of tissue and this is reflected by an enhanced recovery of CaM in hypertensive tissues. Thus, with very diluted tissue samples, the CaM recovery is close to 100% and the endogenous CaM content appears to be slightly lower in hypertensive than in normotensive tissues (Table II). On the other hand, at higher tissue concentrations, the apparent CaM content is significantly higher in hypertensive tissues, presumably because of the interaction between calcium-CaM and the activator (Fig. 1). The increase of CaM activity does not seem to be caused by alterations of CaM but rather by the elevation of its endogenous activator in hypertension.

We also observed an augmentation of this activator in cultured aortic smooth muscle cells derived from SHR. These experiments were performed on cells after the 11th passage, which suggests that the increase of the activator was a consequence of a genetic abnormality and not a sequel of a compensatory hypertrophy mechanism (20).

The general mechanisms by which CaM regulates many enzymes have been reviewed in the literature (30–32). The stimulation of PDE by the activator was Ca\(^{2+}\) and CaM-dependent. The activator appears to affect the affinity of the PDE for CaM rather than its maximal stimulation. Our investigation represents the first demonstration of an activator of CaM in organs and cultured cells that are implicated in the maintenance of hypertension in two different species that have a genetic type of hypertension in common.

Studies to identify this activator are presently being performed in our laboratory. The preliminary results reported here suggest that it is of a lipid nature. Several phospholipids have been demonstrated to activate cyclic nucleotide PDEs (33) but this stimulation is calcium and CaM independent. In addition, phospholipids are known to mimic the effect of CaM on (Ca\(^{2+}\)-Mg\(^{2+}\))-ATPase (34, 35). It is relevant in the context of the present study to underline that the abnormalities of CaM-dependent ATPase have been reported in hypertension (3, 9, 36, 37). We should also mention that in these studies ATPase (found to be decreased in hypertension) was found in membrane fractions in which the cytosolic activator described here would have been removed. It is our imminent goal to ascertain the effect of the cytosolic activator on ATPase activity in hypertension. The phospholipid effects on CaM-dependent PDE reported by Wolff and Brostrom (33) in fact have also been observed to compete with those of CaM, which was not the case for the CaM activator described here.

It has been shown that the insulin-stimulated hydrolysis of a novel phosphatidylinositol-containing glycolipid generates a complex carbohydrate-phosphate substance containing inositol and glucosamine that may stimulate cAMP PDE (38). This glycolipid can be distinguished from the one described herein since the potentiation of CaM-dependent PDE stimulation by the lipid fraction in our experiments is Ca\(^{2+}\) and CaM-dependent and does not appear to be dependent on its hydrolysis into active products. We thus have to first identify the activator of CaM-dependent PDE, which is increased in hypertension, to establish its mechanism of action as well as its putative role in the pathogenesis of hypertension.

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