Effects of Sarcoplasmic Reticulum Calcium Pump Inhibitors on Vascular Smooth Muscle

Chiu-Yin Kwan, Ripan Chaudhary, Xiu-Feng Zheng, Jin Ni, Robert M.K.W. Lee

Abstract: A dysfunctioning of Ca$^{2+}$ pump ATPase in the sarcoplasmic reticulum in vascular smooth muscle has been proposed as a contributing factor for the development of genetic hypertension. In this study, we determined whether in vitro inhibition of the sarcoplasmic reticulum Ca$^{2+}$ pump in vascular smooth muscle tissues and cultured cells isolated from aortas of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats would elicit the known alterations of contractile function and cell growth. We found the following common vascular effects of thapsigargin and cyclopiazonic acid, which are known to be selective inhibitors of sarcoplasmic reticulum Ca$^{2+}$-ATPase in a number of tissues including smooth muscle: (1) Both sarcoplasmic reticulum Ca$^{2+}$ pump inhibitors diminished agonist-induced transient contraction in Ca$^{2+}$-free medium (ie, contraction due to intracellular release of Ca$^{2+}$) and enhanced nifedipine-sensitive contraction on readmission of Ca$^{2+}$ (ie, Ca$^{2+}$ influx via L-type channels); and (2) thapsigargin and cyclopiazonic acid inhibited the attachment of cultured aortic muscle cells to the substrate in a similar degree in both SHR and WKY cells, but SHR cells were more sensitive than WKY cells to the inhibition of cell proliferation by these two agents. The first effect may provide an explanation for several contractile abnormalities known to be associated with elevated cytosolic Ca$^{2+}$ concentration, whereas the second effect suggests that elevation of cytosolic Ca$^{2+}$ in aortic smooth muscle cells is not necessarily associated with or sufficient to account for the accelerated cellular proliferation in SHR. These results, however, further stress the functional importance of impairment of Ca$^{2+}$ regulation in vascular smooth muscle cells in genetic hypertension. (Hypertension. 1994;23[1 suppl]:I-156-I-160.)

Key Words: calcium • muscle, smooth, vascular • sarcoplasmic reticulum

In genetic hypertension, functional defects in Ca$^{2+}$ handling by vascular smooth muscle cells have been proposed to be an etiologically important factor and may represent a major manifestation of a more generalized membrane defect.\(^1,2\) This defect may influence not only the contractile function of vascular smooth muscle in the regulation of blood flow and pressure but also vascular structural changes associated with the development of hypertension. Although an elevated cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) has provided a logical explanation for a number of contractile abnormalities observed in the vascular muscle of spontaneously hypertensive rats (SHR), unequivocal demonstration of the elevation of [Ca$^{2+}]_i$, in vascular smooth muscle cells and the proposed Ca$^{2+}$ mobilization pathways by which the [Ca$^{2+}]_i$ becomes elevated have been matters of continuing dispute.\(^3,4,6\)

Functionally, vascular muscle strips have been found to be more sensitive to the stimuli that affect Ca$^{2+}$ mobilization at the level of either the plasma membrane or sarcoplasmic reticulum (SR).\(^7,8\) Coincidentally, cultured aortic and mesenteric smooth muscle cells tended to grow faster than Wistar-Kyoto (WKY) rat cells under normal cultured conditions.\(^5,9\) Aortic smooth muscle cells from SHR showed an age-dependent change in the basal level of intracellular Ca$^{2+}$.\(^4,5\) An elevated cellular level of Ca$^{2+}$ was found in the platelets and lymphocytes from SHR compared with WKY rats\(^10\) and also in the aortic smooth muscle cells from an experimental model (aortic coarctation) of hypertension.\(^6\) It is therefore possible that there could be a connection between altered Ca$^{2+}$ metabolism and increased vascular smooth muscle proliferation. Previous studies have shown that in a smooth muscle cell line cloned from hamster vas deferens, a highly SR-selective Ca$^{2+}$ pump inhibitor, thapsigargin,\(^11-13\) was found to inhibit DNA synthesis, protein synthesis, and Ca$^{2+}$-ATPase activities.\(^14\) Inhibition of Ca$^{2+}$ sequestration by SR, presumably leading to elevated [Ca$^{2+}]_i$, has been shown to cause an elevation of vascular tone in isolated strip preparations and cell shortening in enzymatically dispersed vascular smooth muscle cells,\(^12\) which appear to mimic the contractile dysfunction observed in preparations from SHR compared with those from WKY rats.\(^15\) Therefore, the primary purpose of this work was to study the effect of two putative SR-selective Ca$^{2+}$ pump inhibitors, thapsigargin and cyclopiazonic acid (CPA), on the attachment and growth of aortic smooth muscle cells isolated from SHR and WKY rats. Thapsigargin is originally derived from plant extracts,\(^11\) and CPA is a toxin derived from fungi. They are structurally unrelated, but both are potent inhibitors of SR Ca$^{2+}$ pump.\(^11,16\) The effects of CPA on the contractility of rat tail artery were also studied and compared with previously reported effects of thapsigargin and CPA on other rat vascular tissues.\(^12,13,17,18\)

Methods

Animals

Male adult Wistar rats (325 to 400 g) were purchased from Charles River Canada, St Constant, Quebec. SHR and nor-
motensive WKY rats were obtained from the rat colonies maintained at McMaster University. These rats originated from the Charles River strains 10 to 18 years ago. Rats were kept and used following government guidelines for the use of experimental animals.

**Contractility Studies**

Wistar rats were killed by stunning and decapitation. The tail arteries were promptly removed and placed in Krebs' physiological solution at pH 7.4 containing (mM/L) NaCl, 119; KCl, 5; CaCl₂, 2.5; MgCl₂, 2; NaHCO₃, 25; NaH₂PO₄, 1; and glucose, 11. Surrounding connective tissues were removed under a dissecting microscope, and vascular tissues were cut into helical strips of approximately 1.5 to 2 mm wide and 30 to 35 mm long. The vascular strips were mounted on a 3-mL organ bath connected to a force transducer (Grass FT03C) and a pen recorder. The organ baths and Krebs' solution were bubbled continuously with 95% O₂ and 5% CO₂ and warmed to 37°C. The solutions in the baths were changed every 20 to 30 minutes. Vascular strips were equilibrated for 20 minutes before the arteries were stretched to approximately 1 g and then were allowed to equilibrate further for at least 90 minutes. Pilot experiments have shown that these helical strips lose their endothelium-mediated responses, based on a lack of acetylcholine (10⁻⁵ mol/L)-induced relaxation in strips precontracted with phenylephrine (10⁻⁶ mol/L). Before data collection, stimulation of the arteries with KCl (60 mM/L) was repeated every 15 to 20 minutes for two to three times or until a reproducible contractile response was obtained. For Ca²⁺-free Krebs', Ca²⁺ was omitted and EGTA (5 to 7.5 x 10⁻⁵ mol/L) was added. For assessment of the status of agonist-sensitive intracellular Ca²⁺ stores, the contractile response to 10⁻³ mol/L phenylephrine was observed shortly after the addition of CPA at concentrations greater than 10⁻⁵ mol/L in about one third of the vascular strip preparations. CPA had little effect on the basal tension of rat tail artery. The most prominent changes were an augmentation of the magnitude of KCl-induced contraction and an attenuation of the rate of relaxation in phenylephrine-induced contraction in the presence of CPA (Table). These effects of CPA were readily reversible.

It is generally accepted that contraction via activation of α₁-adrenergic receptor uses both extracellular and intracellular Ca²⁺. However, in Ca²⁺-free medium, the contraction elicited by α₁-adrenergic receptor agonist is transient, and the intracellular Ca²⁺ store is readily depleted on repeated agonist stimulation. On washout of the agonist, reintroduction of extracellular Ca²⁺ is known to keep the muscle strip quiescent despite a net increase in Ca²⁺ influx, thus suggesting an effective refilling of the empty stores in the superficial SR underneath the plasmalemma. If the empty agonist-sensitive store is refilled, a subsequent application of the agonist in Ca²⁺-free medium will again elicit a compa-

**Aortic Muscle Cell Culture Studies**

Using the explant method, we obtained cultured smooth muscle cells from the thoracic aortas of SHR (systolic blood pressure, 160 to 180 mm Hg) and WKY rats (systolic blood pressure, 110 to 130 mm Hg) at the age of 10 to 12 weeks. Passages of smooth muscle cells from these primary cultures were stored frozen in liquid nitrogen. Cells derived from each rat were propagated separately to compare differences between cells from different animals. For each experiment, cells from SHR and WKY rats in passages 6 to 7 were cultured under identical conditions with Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum and 1% antibiotic-antimycotic solution. At confluency, SHR and WKY cells were trypsinized and seeded into multiwell (24) plates in DMEM with 10% fetal calf serum. The cell numbers were determined using a Coulter Counter with a 100-μm orifice. Dimethyl sulfoxide (DMSO) was used as solvent for thapsigargin and CPA, so equivolume DMSO was also included as a separate control in the cell attachment and proliferation studies.

**Drugs and Chemicals**

All organic chemicals were purchased from Sigma Chemical Co, St Louis, Mo, and GIBCO, Grand Island, NY; all inorganic chemicals were obtained from Fischer, Toronto, Ontario, Canada, and BioRad, Mississauga, Ontario, Canada. Thapsigargin (Sigma) and CPA (Calbiochem, San Diego, Calif) were dissolved in DMSO and added to the bath media in volumes that did not exceed 30 μL. All other drugs were dissolved in deionized and double-distilled water.

**Results**

**Effect of Cyclopiazonic Acid on Vascular Contractility**

When increasing concentrations of CPA up to 10⁻⁴ mol/L were added cumulatively to vascular strips in normal Krebs' solution containing 2.5 mM/L Ca²⁺, only a small, variable, and transient contraction was observed shortly after the addition of CPA at concentrations greater than 10⁻⁵ mol/L in about one third of the vascular strip preparations. CPA had little effect on the basal tension of rat tail artery. The most prominent changes were an augmentation of the magnitude of KCl-induced contraction and an attenuation of the rate of relaxation in phenylephrine-induced contraction in the presence of CPA (Table). These effects of CPA were readily reversible.

**Effects of Cyclopiazonic Acid on Relaxation Time of Contractile Responses to KCl and Phenylephrine After Agonist Washout**

<table>
<thead>
<tr>
<th>Cyclopiazonic Acid (10⁻⁵ mol/L)</th>
<th>Contractile Response</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (60 mM/L)</td>
<td>t₀</td>
<td>27.3±0.8</td>
<td>24.0±2.8</td>
</tr>
<tr>
<td></td>
<td>t₁</td>
<td>60.8±6.8</td>
<td>148.5±16.5*</td>
</tr>
<tr>
<td>Phenylephrine (10⁻⁶ mol/L)</td>
<td>t₀</td>
<td>42.8±8.5</td>
<td>58.8±5.0*</td>
</tr>
<tr>
<td></td>
<td>t₁</td>
<td>135.0±20.3</td>
<td>215.0±38.7*</td>
</tr>
</tbody>
</table>

*Indicates time required to allow 50% relaxation after washout; and t₁, time required for a full relaxation (returning to the basal tension). Values are means±SD. Vascular preparations were challenged with KCl or phenylephrine in the absence of cyclopiazonic acid (CPA). After washout of agonist and after responses fully relaxed to basal tension, CPA was added 15 minutes before reaplication of agonists. Maximal contractile responses to KCl and phenylephrine were 0.85±0.06 and 1.59±0.14 g (n=7), respectively. After CPA treatment, the above maximal response to KCl was augmented to 1.30±0.17 g (n=7) and that to phenylephrine was not altered (1.56±0.13 g, n=5). After CPA washout, corresponding responses to KCl and phenylephrine returned to control level: 0.84±0.10 (n=7) and 1.49±0.17 g (n=5), respectively.

*Significantly different from control.
FIG 1. Typical tracings show effects of cyclopiazonic acid (CPA, 10^{-5} mol/L) on contraction induced by phenylephrine (PE, 10^{-5} mol/L) in Ca^{2+}-free and Ca^{2+}-containing media. Similar results were obtained in four separate experiments. a: Endothelium-denuded rat aortic ring preparation was stimulated with PE in Ca^{2+}-free medium (containing 5x10^{-5} mol/L EGTA). After the transient response, the vascular strip was washed four to five times (W) to remove PE. Reintroduction of 2.5 mmol/L Ca^{2+} to refill Ca^{2+} stores did not induce any contraction over 30 to 45 minutes. Reapplication of PE in Ca^{2+}-free medium restored the transient contraction, b: Similar experiment was carried out as in panel a except that 10^{-5} mol/L CPA was included in the bath chamber before reintroduction of 2.5 mmol/L Ca^{2+}. Addition of Ca^{2+} caused a biphasic contraction. After wash with Ca^{2+}-free medium (W1), reapplication of PE in Ca^{2+}-free medium failed to cause any contractile response. In the same vascular preparation, PE and CPA were effectively washed a few times (W2) to remove PE, and the solution was replaced and washed with Ca^{2+}-containing medium to refill the Ca^{2+} stores for 45 minutes. The preparation was again stimulated with 10^{-5} mol/L PE in Ca^{2+}-free medium. The transient response to PE was restored. c: Experimental protocol remained the same as in panel b except for the inclusion of 10^{-6} mol/L nifedipine (N) after CPA but before Ca^{2+} readmission. Nifedipine apparently inhibited the biphasic response induced by readmission of Ca^{2+}. Similar results were also obtained when 50 mmol/L caffeine was used in place of PE.

Effect of Thapsigargin and Cyclopiazonic Acid on Cell Attachment and Growth

Although the same seeding density of the aortic muscle cells was used in the study of cell growth, a change in cell attachment efficiency could lead to an artifact that mimics a change in cell growth as measured by cell count. Therefore, both cell attachment and cell growth were examined in SHR and WKY aortic cells. The attachment efficiency of smooth muscle cells 24 hours after seeding decreased from approximately 80% to 60% as the concentration of thapsigargin increased from 10^{-8} to 10^{-7} mol/L (mean±SEM, from 78.00±4.14% to 58.00±12.87% in SHR cells, n=4; from 79.50±7.96% to 65.00±11.26% in WKY cells, n=4). The effect of CPA on cell attachment was relatively small as the concentration of CPA was increased from 5x10^{-6} to 15x10^{-6} mol/L in the culture medium (from 75.75±6.04% to 69.00±12.87% in SHR cells, n=4; from 77.00±10.79% to 70.25±12.81% in WKY cells, n=4). DMSO at 0.5% was without any significant effect on the cell attachment. The difference due to different concentrations of thapsigargin and CPA compared with control (DMSO) was significant only for the high concentration of thapsigargin (10^{-7} mol/L) in both SHR and WKY cells (P<.01). Furthermore, there was no difference between SHR and WKY cells in cell attachment under the above conditions by analysis of variance.

Results on cell growth studies indicated that thapsigargin and CPA inhibited the proliferation of both SHR and WKY aortic smooth muscle cells compared with control (0.5% DMSO, Figs 2 and 3, respectively). In either case, the inhibitory effect on cell growth was more prominent in SHR than WKY cells. With the SHR cells, both thapsigargin and CPA significantly inhibited cell growth at various days after plating (P<.001 for thapsigargin and P<.03 for CPA, analysis of variance), whereas in the WKY cells, significant growth inhibition.
FIG 2. Line graphs show effect of thapsigargin on proliferation of aortic smooth muscle cells obtained from four pairs of 10- to 12-week-old Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Cells of the same seeding density from WKY rats and SHR were taken as unity in the measurement of relative cell growth. Data are mean±SEM. o, Control; •, 0.5% dimethyl sulfoxide (DMSO); D, 10⁻⁸ mol/L thapsigargin in 0.5% DMSO; A, 10⁻⁷ mol/L thapsigargin in 0.5% DMSO.

FIG 3. Line graphs show effect of cyclopiazonic acid on proliferation of aortic smooth muscle cells obtained from four pairs of 10- to 12-week-old Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Cells of the same seeding density from WKY rats and SHR were taken as unity in the measurement of relative cell growth. Data are mean±SEM. o, Control; •, 0.5% dimethyl sulfoxide (DMSO); a, 10⁻⁶ mol/L cyclopiazonic acid in 0.5% DMSO.

was observed only with thapsigargin at days 8 and 10 (P<.05) in relation to control (DMSO). The inhibitory effect of thapsigargin was much more potent than that of CPA. A similar inhibitory effect of 15×10⁻⁷ mol/L CPA could be effectively achieved by 10⁻⁴ mol/L thapsigargin. Furthermore, DMSO at 0.5% also significantly inhibited cell growth in SHR and WKY groups, and this effect again was more potent in SHR than WKY cells. Thus, it appears that thapsigargin and CPA affected the proliferation of rat aortic cells from SHR and WKY rats in culture differentially, but this effect was not due to their effect on the attachment of the cells to the substrate.

Discussion

In this work we have clearly demonstrated that CPA caused functional impairment in the contractile properties of rat tail artery. Although we did not measure directly the cytosolic Ca²⁺, the mechanism of action of CPA on rat tail artery, like that on rat aorta,¹⁷ is consistent with its inhibitory effects on the SR Ca²⁺ pump. A major difference between the results obtained in this work and in our previous studies using aorta may reside in the dynamic nature of the responses on Ca²⁺ reintroduction in the presence of CPA. In rat aorta, the responses were more sustained than those obtained in rat tail artery. This may be interpreted as the difference in the efficiency of the plasma membrane Ca²⁺ pump in the removal of Ca²⁺ from the cytosol. Unlike rat aorta, in which SR may play a more prominent role as an intracellular Ca²⁺ sink, in smaller arteries such as rat tail artery, the plasma membrane function may be relatively more prominent such that the accumulated cytosolic Ca²⁺ in the presence of CPA may be more effectively removed via extrusion across the plasma membrane.¹³ It is therefore possible that quantitative or qualitative changes in contractile responses may be present in different vasculatures, or under different experimental conditions, in which the relative contribution of plasmalemmal or SR activities in Ca²⁺ handling may be differentially altered in hypertension. The present findings on vascular smooth muscle indeed underscore some functional alterations of vascular smooth muscle reported in SHR. These alterations include (1) reduced rate of relaxation to KCl- and phenylephrine-induced contraction in vascular smooth muscle as well as in nonvascular smooth muscle,¹² (2) reduced transient contraction of vascular muscle induced by phenylephrine or caffeine in Ca²⁺-free medium,¹⁰ and (3) early onset of contraction induced by readmission of
extracellular Ca\(^{2+}\) to vascular muscle preparation previously depleted of internal Ca\(^{2+}\) by phenylephrine in Ca\(^{2+}\)-free medium.\(^{15}\) Therefore, the fact that the effects of CPA on the contractile function of vascular smooth muscle mimic the contractile abnormalities of vascular muscle in hypertension lends further support to the hypothesis that impaired SR Ca\(^{2+}\) pump may be an important factor in the pathogenesis of hypertension.

Although thapsigargin is generally more potent than CPA in inhibiting SR-selective Ca\(^{2+}\) pump and cellular proliferation, the effect of CPA is considerably rapid\(^{17}\) (<15 minutes as opposed to 1 to 2 hours for thapsigargin\(^{12,13}\)) and readily reversible (thapsigargin is practically irreversible, and its possible long-term side effects have not been assessed). Our finding that smooth muscle cells from SHR were more sensitive to growth inhibition by thapsigargin and CPA than those from WKY rats is indicative of the difference in Ca\(^{2+}\) handling between the cells from these two strains. This could be one of the contributing factors in the difference in vascular smooth muscle cell growth rate observed between SHR and WKY rats.

Acknowledgments

Supported by the Heart and Stroke Foundation of Ontario and the Medical Research Council of Canada.

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Hypertension. 1994;23:I156
doi: 10.1161/01.HYP.23.1_Suppl.I156

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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