Enhanced Responsiveness to Angiotensin II in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats Is Not Associated With Alterations in Protein Kinase C

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This study compares vascular smooth muscle cells from spontaneously hypertensive and normotensive Wistar-Kyoto rats with respect to protein kinase C and intracellular responses to angiotensin II (Ang II). Ang II-induced degradation of polyphosphoinositides and accumulation of inositol di- and tris-phosphates was enhanced (—twofold) in hypertensive-derived cells, without a change (vs. normotensive-derived cells) in half-maximally effective concentrations of Ang II. Intracellular pH (~6.6) was comparable between both cell isolates at quiescence, but alkalinization induced by Ang II, serum, or phorbol ester was greater (Δ 0.1–0.2 pH units) for hypertensive-derived cells. For both cell types, the intracellular pH response to these agonists was prevented in the presence of Na+-H+ exchange inhibitors. S6 kinase activation induced by Ang II was enhanced (—twofold) in hypertensive-derived cells, whereas activation in response to serum or 12-O-tetradecanoylphorbol 13-acetate did not differ significantly between the two cell types. Quantitation of protein kinase C by immunoblotting and [3H]phorbol dibutyrate binding procedures revealed no differences between the two smooth muscle cell isolates (at quiescence or in the presence of serum) with respect to either total amounts or subcellular distribution. Sensitivity of protein kinase C to phorbol ester was apparently also not different between the two cell types, as assessed from dose-dependent (phorbol ester) S6 kinase activation profiles. Phorbol ester caused a similar subcellular redistribution of [3H]phorbol dibutyrate binding in the two cell isolates, but for both, minimal (10%) translocation occurred in response to Ang II. The data suggest that enhanced agonist responsiveness in vascular smooth muscle cells is unlikely to involve alterations in protein kinase C. (Hypertension 1989;14:293–303)

Phospholipase C-mediated breakdown of phosphatidylinositol in the plasma membrane produces two second messenger molecules, namely, inositol 1,4,5-trisphosphate, which promotes release of Ca2+ from intracellular stores, and diacylglycerol, which is required for the physiological activation of protein kinase C (PK-C).1–3 The stimulation of cultured vascular smooth muscle cells (VSMCs) by both growth factors and vasoactive hormones results in a series of transmembrane signals that include phospholipase C activation, Ca2+ mobilization, and activation of PK-C.4–9 These same signals, which are involved in mediating a complicated cascade of cellular events and their coordinated interactions in vivo, are essential to control of VSMC growth and contraction.10–12 The alterations in VSMC growth and contraction, which are of fundamental importance in the development of atherosclerosis and hypertension, may be due to aberrations in signal transduction processes.

Biologically active phorbol esters can mimic the effect of diacylglycerol in the activation of PK-C,3 and since PK-C represents the major cellular phorboid receptor,13–15 the multitude of responses observed with cultured cells exposed to phorbol ester invoke a pivotal role for the PK-C transduction pathway in stimulus-response coupling. Observations that phorbol esters induce a slow but sustained vascular contraction in isolated arteries and that angiotensin II (Ang II) induces a sustained production of diacylglycerol in VSMCs have given rise to the proposal that PK-C activation may be
involved in sustained agonist-induced vasoconstriction.\(^{12,16}\) PK-C is also involved in regulating VSMC growth.\(^{8,17}\) In this regard, we have demonstrated phorbol ester–induced activation of S6 kinase,\(^8\) the stimulation of which is a prerequisite for cell division in quiescent cells.\(^{18}\) Furthermore, phorbol ester induces activation of amiloride-sensitive Na\(^+-\)H\(^+\) exchange, which results in elevation of intracellular pH.\(^{19}\) Such alkalization is intimately associated with both S6 kinase activation and increased proliferation in cultured VSMCs\(^{8,19}\) as well as with increased contractile tone\(^{20}\) in vascular smooth muscle.

Exposure of quiescent VSMCs to Ang II also results in S6 kinase activation\(^8\) and alkalization.\(^{19}\) Thus, these observations together with those relating to stimulation of nuclear proto-oncogene expression by Ang II in VSMCs\(^{21,22}\) have invoked the notion that this vasoconstrictor might also be mitogenic to VSMCs. Ang II has been reported to increase both VSMC size\(^{22}\) and number\(^{23}\) which, in the event of exaggerated and persistent stimulation, could lead to a gradual onset of vascular hypertrophy resulting in a slow rise in peripheral vasoconstriction and arterial pressure. The blood pressure–lowering effect of angiotensin converting enzyme inhibition has provided indirect evidence for a role of Ang II in the pathophysiology of hypertension.\(^{24}\) Direct evidence to support such a role has been obtained from studies on cultured VSMCs from genetically hypertensive rats in which both Ang II–stimulated Ca\(^{2+}\) flux and Na\(^+-\)H\(^+\) exchange\(^{25}\) were found to be enhanced relative to VSMCs from their normotensive controls. The involvement of PK-C in mediating such increased responsiveness to Ang II in VSMCs from spontaneously hypertensive rats (SHR) has not been studied.

This study further investigates metabolic responsiveness to Ang II in cultured aortic VSMCs from SHR and normotensive Wistar-Kyoto (WKY) rats. PK-C levels and translocation responses were also determined in the cells, and we have attempted to evaluate the role of this enzyme in hypertension.

**Materials and Methods**

**Materials**

SHR and WKY rats (male, aged 20 weeks, and weight 200–250 g) were obtained from the inbred Wistar strain described by Okamoto and Aoki\(^{28}\) and supplied via Madoerin AG, Fuellinsdorf, Switzerland. Blood pressures were measured (tail-cuff procedure) before rats were killed, and typical values (mean±SD, \(n=6\)) obtained were 215±6 mm Hg for SHR and 130±5 mm Hg for WKY rats. The chemicals and media for tissue culture were obtained from Gibco AG, Basel, Switzerland, with the exception of fetal calf serum (FCS), which was purchased from Fakola AG, Basel, Switzerland. All the radioisotopes and radiolabeled compounds used in this study were obtained through Rahn and Co. (Amer-
SHR/WKY rat-derived cells were performed as described previously. For activation, cells were incubated at 37°C for 15 minutes in freshly replaced serum-free medium without additions or with various concentrations of either Ang II, 12-0-tetradecanoylphorbol 13-acetate (TPA) or 10% FCS as positive control. Activations were terminated by rapid washing of cells with 3×2 ml aliquots of cold extraction buffer (20 mM HEPES, 15 mM MgCl2, 20 mM EGTA, 1 mM dithiothreitol, and 80 mM β-glycerol phosphate, pH 7.3), followed by scraping cell layers into 300 μl extraction buffer containing 0.2% vol/vol Triton X-100 and 5 mM phenylmethylsulfonylfluoride. Cell lysates were collected after centrifugation for 15 minutes at 11,000g at 5°C, and samples were stored at −70°C until use. S6 kinase phosphorylation assays (specific activity of [8-32P]ATP at ~2×10⁶ disintegrations per minute (dpm)/pmol) were performed using 40S ribosomal subunits as substrate as described previously.9 Stimulation of cells with 10% FCS resulted in the incorporation of between 4 and 4.8 pmol PO4/10⁶ cells, whereas unstimulated cells (serum-free) exhibited values of 0.42–0.72 pmol PO/10⁶ cells. Neither of these values differed significantly between SHR- and WKY rat-derived VSMCs.

Phosphoinositide metabolism. Confluent VSMCs were rendered quiescent and inositol lipids prelabeled to equilibrium by incubation in serum-free and inositol-free medium containing myo[2-3H]-inositol (5 μCi/ml) for 48 hours. Thereafter, cells were washed three times with phosphate buffered saline (PBS) before addition of 1 ml isotonic PBS containing 4.4-0.72 pmol PO4/10⁶ cells. Neither of these values differed significantly between SHR- and WKY rat-derived VSMCs.

Measurement of intracellular pH. Intracellular pH (pH) was measured with the weak acid 5,5-dimethyl[2,4-14C]-2,4-oxazolidine-dione (DMO) by procedures essentially as described by Mendoza and Rozengurt. The electrolyte solution contained 50 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.9 mM MgCl2, 25 mM glucose, 90 mM choline chloride, and 30 mM HEPES-Tris (pH 7.2). In some experiments choline chloride was omitted and replaced by NaCl to give physiological NaCl (140 mM) concentrations (see Results). Confluent, quiescent VSMCs were exposed to Ang II (0.1 μM), TPA (0.1 μM), or FCS (10%) for 45 minutes at 37°C before addition of [14C]DMO (final concentration 150 μM, 1.5–2.0×10⁶ dpm/well). Values for “trapped” extracellular [14C]DMO were routinely subtracted from the experimental points. Intracellular volumes (μl/mg protein) were determined by [14C]urea and were 7.19±1.06 (n=4) and 7.52±0.67 (n=4) for cells derived from SHR and WKY rats, respectively. Protein concentrations were measured after solubilization of cells in 0.2 NaOH/0.2% Triton X-100. Intracellular pH was calculated by using the formula of Waddell and Butler.32

[1H]4β-Phorbol 12,13-dibutyrate binding to intact cells. Confluent VSMCs were rendered quiescent before measurement of [1H]PDBu binding. Intact VSMCs (2–4×10⁶ cells/well) were incubated in 1.0 ml MEM containing 1.0 mg bovine serum albumin/ml and buffered (pH 7.3) with 20 mM TES-NaOH, 20 mM HEPES-NaOH. [1H]PDBu was added at varying concentrations (0.5–40.0 nM), and a parallel series of wells also contained 10 μM unlabeled PDBu for determination of nonspecific binding. Binding was performed at 37°C for 45 minutes; then [1H]PDBu-containing medium was aspirated and wells washed with 3×4 ml aliquots of ice-cold buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10 mM Mg (NO3)2, 1 mM CaCl2, and 1 mg bovine serum albumin/ml. Cell layers were solubilized in 1.0 ml 1% sodium dodecyl sulfate (SDS) and bound [1H]PDBu was determined by liquid scintillation counting. Specific binding was estimated as total bound minus that bound in the presence of 10 μM PDBu. Binding parameters were obtained for each individual experiment by computerized weighted nonlinear curve fitting analysis.33

[1H]4β-Phorbol 12,13-dibutyrate binding to cell fractions. Nonquiescent, confluent VSMCs were washed with PBS at 4°C before they were harvested and sonicated in extraction buffer (PKB) containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 20 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonylfluoride. A fraction of the sonicate (homogenate) was withdrawn, and the remainder was centrifuged at 100,000g for 30 minutes at 4°C. Supernatants (cytosol) were withdrawn before suspension and sonication of pellets (membranes) in PKB. All samples were stored at −70°C until use. [1H]PDBu binding to each fraction was performed essentially as described previously.34 Sample aliquots (50–100 μg protein) were incubated (overnight at 4°C with vigorous shaking) in a reaction mixture (250 μl) containing 20 mM Tris-HCl (pH 7.4), 10 mM Mg(NO3)2, 1 mM CaCl2, 400 μg/ml phosphatidylserine, 4 mg bovine serum albumin/ml, and various concentrations of [1H]PDBu (0.5–50 nM), and in the presence or absence of 5 μM unlabeled PDBu (to determine nonspecific binding; usually less than 10% of specific [1H]PDBu binding). Thereafter [1H]PDBu binding was determined by filtration through Whatman GF/C filters (Whatman, England) and washing with 4×3 ml aliquots of ice-cold buffer containing 20 mM Tris-HCl (pH 7.4).
10 mM Mg(NO₃)₂, 1 mM CaCl₂. Bound radioactivity was quantitated and specific binding parameters determined by weighted nonlinear curve fitting analysis.33

In translocation experiments, VSMCs were grown to confluence in 100 mm petri dishes and then rendered quiescent before exposure to TPA (10 nM) or various concentrations of Ang II for 5 minutes at 37° C. Dishes were placed on ice, aspirated with medium, and cells washed rapidly three times with PBS. Cells were harvested into 700 µl PKB, and cell fractions were prepared as described above. [³H]PDBu binding in homogenates, cytosols, and membranes was determined as described above except that only a single saturating concentration of [³H]PDBu (50 nM) was used. Specific binding was determined, and total [³H]PDBu bound in each fraction was estimated after appropriate corrections for extraction and assay volumes were made. Data express the percentage distribution of [³H]PDBu between cytosol and membrane fractions where that bound in the homogenate fractions was taken to represent 100%.

Quantitation of total cell protein kinase C. Confluent, quiescent VSMCs were washed with PBS before addition of 75 µl SDS-solubilizing buffer (10 mM Tris-HCl [pH 7.0], 3% SDS, 2% β-mercaptoethanol, 2 mM EDTA, 10% glycerol) to three wells, and 1.0 ml 0.2N NaOH, 0.2% Triton-X-100 to the remaining wells. Total cell SDS extracts were transferred to Eppendorf tubes, heated at 95° C for 5 minutes and then subjected to SDS-polyacrylamide (10%) gel electrophoresis (~50-75 µg protein/slot) followed by electrophoretic transfer to nitrocellulose membranes (Bio-Rad Labs., Richmond, California). Membranes were processed for immunoblotting exactly as described using monoclonal antibody to PK-C (clone MCS, Amersham) and Iodine-125-labeled (0.2 µCi/ml) sheep-anti-mouse second antibody. Clone MCS has been reported to recognize α and β isoforms of PK-C. Immune detectable PK-C (Mr 80 kDa) was quantitated after it was located by autoradiography, then the labelled area was excised from the membranes and counted in a Gamma counter. For comparative (SHR vs. WKY rats) purposes, all values were normalized with respect to protein loaded in each SDS-polyacrylamide gel slot. Proteins were determined in NaOH/Triton-X-100 solubilized samples by the method of Bradford and with bovine serum albumin as standard.

Statistical Analysis

Unless otherwise stated values are given as mean±SD where n=number of separate experiments performed. Statistical significance was analyzed using Student's t test for unpaired data.

Results

Phosphoinositide Metabolism and Intracellular pH

To compare the efficacy of Ang II in promoting phosphoinositide breakdown in VSMCs from SHR and WKY rats, a 2-minute exposure period was found to be optimal for reproducible and accurate quantitation of dose-dependent changes in all phosphoinositides and inositol phosphate fractions. SHR-derived VSMCs exhibited an amplified phosphoinositide catabolic response as evidenced by their elevated (vs. WKY rat VSMCs) accumulated levels of inositol trisphosphate and bisphosphate (Figure 1). The significance of difference between SHR and WKY rat derived VSMCs reached threshold levels (p<0.05) at 1 nM Ang II and at saturating concentrations of Ang II was p<0.001. After a 2-minute exposure to Ang II, accumulated levels of inositol monophosphate were only slightly greater in SHR-derived VSMCs than in WKY rat VSMCs (both at 0.8-1.08X10⁶ cells/well) for Gro-PIns, Ins-P, Ins-P₂, and Ins-P₃, respectively, were as follows: SHR, 3,057±948, 11,103±4,176, 1,463±799, and 1,470±249; WKY rats, 2,679±984, 10,368±4,099, 1,212±595, and 1,117±342.

FIGURE 1. Dose-dependent accumulation of inositol phosphates in response to angiotensin II (Ang II) in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. [³H]myo-inositol-prelabeled and quiescent VSMC from SHR (○) and WKY rats (□) (both at 0.8-1.08X10⁶ cells/well) were treated for 2 minutes at 37° C with the indicated concentrations of Ang II (and in the presence of 30 mM LiCl). Tritium content was quantitated after extraction and chromatographic resolution of glycerol-phosphoinositol (Gro-PIns), inositol monophosphate (Ins-P), inositol diphosphate (Ins-P₂), and inositol trisphosphate (Ins-P₃). All experimental details are given under Materials and Methods. Data (mean±SD, n=4) express the percentage of initial tritium content (in the absence of Ang II), which was taken as 100% for each inositol phosphate. The absolute initial values (dpm/0.8-1.08X10⁶ cells) for Gro-PIns, Ins-P, Ins-P₂, and Ins-P₃, respectively, were as follows: SHR, 3,057±948, 11,103±4,176, 1,463±799, and 1,470±249; WKY rats, 2,679±984, 10,368±4,099, 1,212±595, and 1,117±342.
phosphate (percent of unstimulated tritium content: SHR, 73.4 ± 7.0; WKY, 86.6 ± 2.8). The concentrations of Ang II required to elicit half-maximal inositol phosphate accumulation (Figure 1) and deg- radation of phosphoinositide (complete data not shown) were comparable and did not differ between SHR and WKY rat derived VSMCs (~4–8 nM). Enhanced phosphoinositide responsiveness to Ang II in SHR VSMCs was evident not only from dose-response profiles (Figure 1, 2 minutes) but also from kinetic profiles (10 M Ang II, 15 seconds to 5 minutes; compared in two paired SHR/WKY rat isolates, data not shown).

There was no difference in pH_i of VSMCs from the two animal sources (SHR/WKY rats) when measured under serum-free conditions (quiescence) (Table 1). After stimulation by FCS (10%), TPA (100 nM), or Ang II (100 nM), significant (p at least <0.05) alkalinization was measured in both VSMC isolates, but the increase in pH_i (ΔpH_i) was twofold greater for SHR-derived VSMCs than those from WKY rats (Table 1). Inclusion of Na^+ -H^+ exchange inhibitors such as amiloride (0.5 mM), dimethylamiloride (DMA) (10 μM), or ethylisopropylamiloride (EIPA, 1 μM) completely abolished the alkalinization response of both cell types to Ang II, FCS, and TPA (pH_i values ranged between 6.2 and 6.5).

The present pH_i values (Table 1) are somewhat lower than those (~7.08) reported by Berk et al in their study on Na^+ -H^+ exchange in VSMC. This discrepancy is probably because we have used quiescent VSMCs (serum-deprived for 48 hours), a lower external pH, and low Na^+ in the electrolyte solution, all conditions that have pH_i-lowering effects. Measurement of pH_i in quiescent VSMCs under physiological NaCl (140 mM) concentrations yielded slightly higher values (±0.05–0.1 units) than those in 50 mM NaCl/90 mM choline chloride concentrations. Under these conditions quiescent SHR and WKY rat VSMCs also did not differ with respect to basal pH_i, and SHR VSMCs still exhibited the greater alkalinization response to the agonists tested (Table 1).

**Phorbol Ester Binding and Protein Kinase C**

Estimation of PK-C by saturation analysis of [3H]PDBu binding at 37° C to intact quiescent VSMC (Figure 2) and Scatchard transformation of data obtained for specifically bound [3H]PDBu (inset, Figure 2) indicated that VSMCs from SHR and WKY rats bound comparable amounts of the phor- bol ester (0.43 ± 0.07 and 0.41 ± 0.02 pmol/10^6 cells, respectively). The linearity of Scatchard plots indicated one class of [3H]PDBu receptors with comparable affinities for PDBu between SHR and WKY rat derived VSMCs. The two cell types also did not differ with respect to [3H]PDBu binding kinetics (data not shown). [3H]PDBu binding parameters from individually analyzed saturation experiments are summarized in Table 2. [3H]PDBu binding parameters determined after 3 hours of incubation at 4° C were comparable with those obtained after 45 minutes at 37° C (compared in two pairs of SHR/WKY rat VSMC isolates, data not shown). Immunoblot analysis of PK-C in total cell extracts (at quiescence), using a commercial monoclonal antibody to PK-C, revealed comparable amounts of immunoreactive polypeptide (M, 80 kDa) in VSMCs from SHR (14.4 ± 1.25 cpm/mg total protein, n = 5) and WKY rats (13.3 ± 1.38 cpm/mg total protein, n = 5). An autoradiogram indicating immunoreactivity specific- ity of clone MC5 antibody and the comparable immunoreactivity between SHR/WKY cell extracts is given in Figure 3.
FIGURE 2. Binding of [3H]4β-phorbol 12,13-dibutyrate (PDBu) to intact vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Binding of [3H]PDBu to intact, quiescent VSMCs from SHR (•) and WKY rats (○) was performed as described in Materials and Methods. Values (mean of triplicate determinations) shown for non-specific (dotted line) and specific (solid line) binding were obtained from a representative matched pair (SHR/WKY rat) of VSMC isolates. Binding parameters obtained from five matched pairs of VSMC isolates are summarized in Table 2. The Scatchard plot inset was obtained by transformation of data for specific [3H]PDBu binding in the exhibited saturation profile.

To determine whether the distribution of PK-C in membrane and cytosolic compartments might be different between SHR and WKY rat VSMCs, [3H]PDBu binding in the subcellular fractions was determined (data summarized in Table 2). The distribution of [3H]PDBu binding (percent of that bound in homogenate) between membranes (60%) and cytosol (40%) was similar for the two sources (SHR and WKY rats) of VSMCs when cultured under normal growth (10% FCS) conditions. Quantitatively, the amounts of [3H]PDBu bound (pmol/mg protein) in either membrane or cytosol were not different between SHR- and WKY rat-derived VSMCs. A similar observation was made for [3H]PDBu binding in total cell homogenates, although the amounts of phorbol ester bound in these experiments (pmol/mg) were markedly less than the analogous values calculated for intact, quiescent VSMCs. Such a difference may reflect a partial down-regulation/proteolysis of PK-C (analogous to its down-regulation in nonquiescent VSMCs).

![Graph showing PDBu binding to VSMCs from SHR and WKY rats](image)

**TABLE 2.** [3H]4β-Phorbol 12,13-dibutyrate Binding Parameters in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats and Wistar-Kyoto Rats

<table>
<thead>
<tr>
<th>Cell/fraction</th>
<th>WKY</th>
<th>SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>3.97±0.96</td>
<td>4.24±0.88</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.30±0.31</td>
<td>1.58±0.30</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.56±0.10</td>
<td>0.59±0.16</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranes</td>
<td>0.87±0.11</td>
<td>0.92±0.12</td>
</tr>
<tr>
<td>(n=10)</td>
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[3H]4β-phorbol 12,13-dibutyrate (PDBu) binding either to intact, quiescent vascular smooth muscle cells (VSMC) (see Figure 2) or in homogenate, membrane, and cytosol fractions from nonquiescent VSMC was performed as described under Materials and Methods. Each saturation profile (where n = number of separate experiments on pairs of spontaneously hypertensive rats (SHR)/Wistar-Kyoto (WKY) rat) VSMC was individually analyzed. Values given represent mean±SD. *Indicate significant differences (p<0.05) between SHR and WKY rats. N.D., not determined; Bmax, maximum binding capacity; KD, dissociation constant.
ous to that induced by long-term treatment with phorbol ester\(^2\) in those VSMCs chronically exposed to serum-containing growth factors and hormones. Within each cell type, the affinity for \(^{[3]H}\)PDBu was comparable between membrane and cytosolic fractions. However, the calculated dissociation constants (\(K_d\)) were lower (\(p<0.05\)) for SHR VSMCs than for WKY rat VSMCs (Table 2). For both cell types the \(K_d\) values obtained in membranes and cytosol fractions (from nonquiescent VSMCs) were lower than those found in quiescent, intact VSMCs as has been observed for intact mammary carcinoma cells (5–12 nM) and cytosolic fractions thereof (0.5–1.2 nM).\(^4\)

TPA and a variety of growth factors that stimulate phosphoinositide hydrolysis and diacylglycerol formation induce translocation of PK-C from the cytosol to membranes, and this is believed to reflect intracellular activation of the enzyme.\(^4,4^2\) To investigate whether Ang II can promote PK-C translocation VSMCs were rendered quiescent before exposure to this vasoconstrictor. In such quiescent VSMCs, the subcellular distribution of \(^{[3]H}\)PDBu binding was comparable between SHR- and WKY rat–derived cells (Figure 4) (membranes ~30–40% and cytosol ~60–70%). After a 5-minute exposure to various concentrations of Ang II, at which time Ang II–stimulated diacylglycerol formation is maximal,\(^4\) a slight dose-dependent trend for translocation was observed (Figure 4). However, for neither cytosol nor membrane fractions were the values (percent) significantly different from those in untreated quiescent VSMCs. This contrasts with the effect of TPA (10 nM), which decreased cytosolic \(^{[3]H}\)PDBu binding by ~30% and concomitantly increased (~30%) membrane phorbol ester binding (Figure 4). SHR- and WKY rat–derived VSMCs did not differ with respect to either stimulation of translocation by 10 nM TPA or their minor translocation response to Ang II. In a further series of experiments (data not shown) that investigated a possible kinetic dependence of \(^{[3]H}\)PDBu binding transposition in response to Ang II (10\(^{-7}\) M), quiescent VSMCs from neither SHR nor WKY rats exhibited a significant translocation response after 1, 2, 5, 15, and 30 minutes of exposure to TPA and remained stable for up to the 30-minute treatment period examined (data not shown).

**Activation of S6 Kinase**

S6 kinase activation can be mediated by both PK-C and intrinsic receptor tyrosine–kinase coupled pathways.\(^5,1^8\) Ang II and TPA elicit activation of S6 kinase by PK-C–independent and -dependent mechanisms, respectively.\(^5\) The S6 kinase response to Ang II and TPA was examined in quiescent SHR- and WKY rat–derived VSMCs (Figure 5). Activation of S6 kinase in response to TPA was comparable between the two cell types (Figure 5B). On the other hand, VSMCs from SHR exhibited an exaggerated S6 kinase activation in response to Ang II compared with WKY rat–derived VSMCs, but this did not involve an altered affinity (half-maximally effective concentration 5 nM) for Ang II (Figure 5A). These data would indicate that the PK-C–dependent pathway for S6 kinase activation (by TPA) is comparable between SHR and WKY rat VSMCs, whereas differences between the two cell types are evident for the PK-C–independent pathway of S6 kinase stimulation.

Although S6 kinase activation (polysome formation) is dependent on alkalization, it is most sensitive to conditions that increase pH by Δ 0.2 units in that further alkalization does not significantly increase polysome formation.\(^3^9,4^0\) Thus, the present data demonstrating comparable S6 kinase activation (~4 pmol PO\(_4\) incorporated/10\(^6\) cells) for quiescent VSMCs (SHR and WKY rats) stimulated by 10% FCS (see data under Materials and Methods) or 10\(^{-7}\) M TPA (Figure 5B) and for SHR VSMCs stimulated by 10\(^{-7}\) M Ang II (Figure 5A) are consistent with the data on pH\(_1\) (Table 1) where the pH\(_1\) for the above ranged from 0.17 to 0.4 units. For

![Graph](http://hyper.ahajournals.org/)
pared VSMC lysates for S6 kinase activation. There is much evidence that SHR-derived VSMCs were found to be amplified in SHR-derived VSMCs response of VSMCs to Ang II is mediated both by intracellular stores and Ca2+ entry through receptor-operated Ca2+ channels. One other study (using quin-2) failed to demonstrate an increased Ca response to Ang II. The Ca2+ uptake in SHR VSMCs compared with WKY rat VSMCs. Our observations agree with other preliminary studies reporting greater Ang II–induced changes in pH, (using the fluorescent probe BCECF) in passaged aortic VSMCs from 10–12-week-old SHR, and increased Ang II–stimulated inositol phosphate accumulation in primary cultures of aortic VSMCs from adult SHR. In these studies, the increase in intracellular Ca2+ (assessed either by fura-2 or 44Ca2+ uptake) in response to Ang II was also enhanced in SHR VSMCs compared with WKY rat VSMCs. One other study (using quin-2) failed to demonstrate an increased Ca2+ response to Ang II. The Ca2+ response of VSMCs to Ang II is mediated both by inositol trisphosphate–promoted release of Ca2+ from intracellular stores and Ca2+ entry through receptor-operated Ca2+ channels. There is much evidence to indicate that receptor-operated Ca2+ channels are altered in SHR vessels (see Reference 47 for review), and this together with our observation of increased inositol trisphosphate formation in response to Ang II could account for the above findings, with respect to an increased intracellular Ca2+ response in SHR VSMCs.

Enhanced phosphoinositide responsiveness to Ang II may reflect altered Ang II receptor properties. However, Schifrin et al have demonstrated vascular Ang II receptors (in mesenteric vessels) to be different between SHR and WKY rats only at the prehypertensive (4–6 weeks) phase (increased number in SHR) and not in older (12 weeks, with development of high blood pressure in SHR) rats. Differences in affinity for Ang II were not evident at any time. It has been hypothesized that the increased vascular reactivity of SHR to Ang II during the established phase of hypertension depends on post-receptor mechanisms. Increased phospholipase C activity has been reported in various tissues of SHR including the arterial wall, erythrocytes, and platelets. Such an aberration could account for our present findings with respect to phosphoinositide metabolism. An alternative explanation may reside in changes at the level of guanine nucleotide binding (G) proteins since these are believed to couple phospholipases to receptors or to regulate phospholipase C activity. However, associations between hypertension and alterations in G proteins cannot presently be made because of a relative paucity of definitive information concerning the specificity of G protein involvement in agonist-induced transmembrane signalling.
bol ester. Such data would imply equivalent PK-C-mediated activation of Na\(^+\)-H\(^+\) exchange in both VSMC isolates. However, TPA, Ang II, and serum-induced alkalinization were all significantly greater for SHR VSMCs, suggesting that increased Na\(^+\)-H\(^+\) exchange in these cells is not solely dependent on direct activation by PK-C but could also involve either some process distal to activation of this enzyme or some moiety of the Na\(^+\)-H\(^+\) antiporter itself. Moreover, Berk et al\(^{38}\) have clearly demonstrated that Na\(^+\)-H\(^+\) exchange in VSMCs can be stimulated by both PK-C-dependent and PK-C-independent pathways.

There is ample evidence documenting association between cytosol to membrane translocation of PK-C and its intracellular activation.\(^{3,15,42,43}\) The effect of Ang II on subcellular translocation of [\(^{3}H\)]PDBu binding was minimal (–10% at maximal doses), and not different between SHR and WKY rat VSMCs. This apparent ineffectiveness of Ang II on translocation contrasts with the different subcellular [\(^{3}H\)]PDBu binding distributions observed between VSMCs at quiescence and those either stimulated by TPA or continuously cultured in the presence of 10% FCS. The latter data furthermore suggest that SHR and WKY rat VSMCs exhibit comparable PK-C translocation responses to both TPA and FCS. Although our findings with respect to Ang II–induced redistribution of [\(^{3}H\)]PDBu binding imply only minor activation of PK-C (–10% of total), it is possible that Ang II translocates a specific isofrom of PK-C. In such a case, the extent of PK-C activation (and cellular effects thereof) cannot be assumed to relate directly to redistribution of total cellular [\(^{3}H\)]PDBu binding. Nevertheless, it is important to note that down-regulation of PK-C in VSMCs by long-term exposure to phorbol ester (which negates subsequent responsiveness to phorbol) does not inhibit either Ang II–promoted Na\(^+\)-H\(^+\) exchange\(^{38,41-54}\) or Ang II–induced S6 kinase activation\(^{8}\) in VSMCs. Furthermore, inclusion of the PK-C inhibitor H7, failed to inhibit Ang II–stimulated alkalinization.\(^{38}\) Such data would indicate that in spite of an amplified Ang II–stimulated phosphoinositide breakdown and intracellular Ca\(^{2+}\) responses in SHR VSMCs (present study and References 23 and 25), coupled with a sustained diacylglycerol response to Ang II in VSMCs,\(^{24}\) PK-C is not necessarily involved in mediating the observed differential (SHR vs. WKY rats) pH\(_{i}\) and S6 kinase activation responses to Ang II.

An intrinsic Ang II–receptor kinase activity, analogous to that of growth factor and other hormone receptors, has been postulated to account for the PK-C–independent effects of Ang II on Na\(^+\)-H\(^+\) exchange.\(^{38,54}\) The amiloride sensitivity of Ang II–induced S6 kinase activation,\(^{8}\) together with the fact that phosphorylation of S6 kinase itself is necessary for enzyme activity,\(^{44}\) also invokes the possibility of intrinsic receptor kinase activity in mediating PK-C–independent S6 kinase activation responses to Ang II. At present, there is no real evidence for intrinsic Ang II–receptor kinase activity, but such a postulate is quite plausible given that some growth factors (e.g., epidermal growth factor and platelet-derived growth factor) have now been found to induce vasoconstriction\(^{6}\) whereas not only Ang II but also vasopressin and catecholamines can exert trophic actions on cultured VSMCs (reviewed in Reference 55). Alternative processes controlling Na\(^+\)-H\(^+\) exchange (and thus S6 kinase because of its dependence on alkalinization for activation\(^{8}\) include transmethylation and those pathways linked to Ca\(^{2+}\) calmodulin or guanosine triphosphate binding proteins.\(^{20}\)

The increased Ang II–induced S6 kinase activation response in SHR VSMCs (vs. WKY rat VSMCs) may be an important mechanism for VSMC hypertrophy in hypertension. Activation of S6 kinase is an essential event for the reactivation of protein synthesis and subsequent DNA synthesis\(^{18}\) in quiescent cells. Ang II has been reported to increase synthesis of protein, RNA, and DNA in VSMCs,\(^{22}\) and the enhanced Ang II–induced [\(^{3}H\)]uridine and [\(^{3}H\)]thymidine incorporation reported for SHR VSMCs (vs. WKY rat VSMCs)\(^{23}\) may thus be a consequence of their greater S6 kinase activation response to Ang II. Nevertheless, the influence of Ang II on VSMC proliferation remains unclear. In our laboratory, Ang II alone (under serum-free conditions) did not exert mitogenic effects, as assessed by cellular enumeration or nuclear labeling, in VSMCs from either SHR\(^{8}\) or WKY rats (unpublished observation). On the other hand, Ang II has been reported either to increase VSMC size, but not cell number,\(^{25}\) or to increase VSMC number to a greater extent in SHR than WKY rats.\(^{25}\) It is not clear from either of these reports whether VSMCs had been rendered quiescent before experimentation or whether the effects of Ang II were determined under serum-free conditions.

This study provides further evidence to support enhanced metabolic responsiveness to Ang II in hypertension. Amplified pH\(_{i}\), phosphoinositide, and S6 kinase activation in VSMCs are likely to significantly influence both contractile and growth responses of the vasculature. Although PK-C is undoubtedly an important mediator of these biological responses, the data presented strongly suggest that deregulation of agonist-induced intracellular responses in VSMCs from SHR is unlikely to directly involve PK-C.

References
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