Insulin-Induced Ca$^{2+}$ Transport Is Altered in Vascular Smooth Muscle Cells of Spontaneously Hypertensive Rats

R.M. Touyz, E.L. Schiffrin

Abstract Intracellular calcium may be a mediator of insulin action in vascular smooth muscle cells. This study investigates effects of physiological concentrations of insulin on intracellular free calcium concentrations in primary unpassaged vascular smooth muscle cells derived from 3- and 17-week-old normotensive rats (Wistar and Wistar-Kyoto) and spontaneously hypertensive rats (SHR). Underlying mechanisms responsible for insulin-evoked calcium responses were also studied. Basal calcium was significantly higher in 17-week SHR cells (134±8 nmol/L) compared with cells from Wistar-Kyoto (98±12 nmol/L) and Wistar (99±10 nmol/L) rats. Insulin (70 µU/mL) significantly increased calcium in all cells. Responses from 3-week rat cells were similar. The increase was amplified in 17-week SHR cells (177±7 nmol/L) compared with Wistar-Kyoto (130±14 nmol/L) and Wistar (132±16 nmol/L) cells. Genistein (0.1 µmol/L) and tyrphostin 23 (0.1 µmol/L) (tyrosine kinase inhibitors) completely abolished insulin-induced calcium effects. Stimulatory effects of insulin were significantly inhibited by 0.1 µmol/L diltiazem, staurosporine, calphostin C, and thapsigargin. The inhibitory effects of diltiazem (calcium channel antagonist) and the protein kinase C inhibitors staurosporine and calphostin C were significantly lower in cells from hypertensive compared with those from normotensive rats. Calcium recovery after insulin administration was delayed in SHR cells. In conclusion, insulin increases vascular smooth muscle cell calcium concentrations, possibly via calcium channel activation, protein kinase C-mediated mechanisms, and intracellular calcium mobilization. Alterations of these pathways as well as impaired calcium recovery to baseline may be associated with increased insulin-sensitive calcium responses in cells from SHR. These insulin-related calcium effects may be an expression of abnormalities in calcium handling rather than of insulin action and may be secondary manifestations rather than a primary event in hypertension. (Hypertension. 1994;23[part 2]:931-935.)

Key Words • calcium • protein kinase C • calcium channels • Ca$^{2+}$-transporting ATPase • protein-tyrosine kinase

Insulin influences many transmembrane cation transport systems, including Ca$^{2+}$-ATPase activity, Na$^+$,K$^+$-ATPase, Na$^+$-H$^+$ antiport, and Ca$^{2+}$-Na$^+$ exchange systems.1-3 By modulating various membrane transporters, insulin has been found to alter intracellular free calcium concentrations ([Ca$^{2+}$]$_i$) and may influence the regulation of intracellular calcium homeostasis.4,5 [Ca$^{2+}$]$_i$ is an important determinant of vascular smooth muscle contractility.6 Insulin may influence vascular smooth muscle function by altering the movement of Ca$^{2+}$ into vascular smooth muscle cells (VSMCs) or by affecting Ca$^{2+}$ mobilization from intracellular stores. Thus, insulin could play a role in the contractile properties of VSMCs and in the regulation of blood pressure. Recent reports have suggested that impaired cellular responses to insulin are associated with increased vascular smooth muscle tone and hypertension.7,8 These effects have been attributed to changes in [Ca$^{2+}$]$_i$. A recent study demonstrated that in cultured VSMCs from spontaneously hypertensive rats (SHR), insulin-sensitive Ca$^{2+}$ transport was found to be decreased.9 Although insulin may alter [Ca$^{2+}$]$_i$, the mechanisms by which this occurs remain unclear.

This study assesses the effects of physiological concentrations of insulin on [Ca$^{2+}$]$_i$ in primary unpassaged VSMCs derived from young and adult normotensive and hypertensive rats and attempts to elucidate the possible mechanisms underlying insulin-induced [Ca$^{2+}$]$_i$ responses in VSMCs.

Methods

Materials

All chemicals were of the highest reagent grade available. Insulin (human, recombinant) was obtained from Sigma Chemical Co; fura-2 acetoxymethyl ester (fura-2, AM) and pluronic F-127 were from Molecular Probes. Thapsigargin, tyrphostin 23, calphostin C, genistein, and staurosporine were from Calbiochem. The other chemicals were obtained from ICN, Fischer Scientific Co, and BDH Inc.

Cell Preparations

Male Wistar-Kyoto (WKY) and Wistar rats and SHR were studied at 3 and 17 weeks of age. Systolic blood pressure was recorded in pretrained, conscious rats by the tail-cuff method 1 to 2 days before experimentation. Rats were killed by decapitation. Primary unpassaged cells were derived from the mesenteric arteries. The cells were prepared according to previously described techniques.10 Mesenteric arteries were cleaned of all connective tissue. Smooth muscle cells were dissociated by digestion of arteriolar arcades with 0.12 mg/mL elastase, 2 mg/mL collagenase (type 1), 0.36 mg/mL soybean trypsin inhibitor, 2 mg/mL bovine serum albumin, and 100 µg/mL gentamycin in Ham's F-12 medium for 1 hour at 37°C. The tissue was filtered through a 100-µm nylon mesh and the cell suspension centrifuged at 200g and resuspended in Dul-
becco's modified Eagle medium (DMEM) containing 10% heat-inactivated calf serum, 2 mmol/L L-glutamine, 20 mmol/L HEPES (pH 7.4), 10 000 U penicillin, and 10 000 μg/mL streptomycin. Cells were grown on round glass coverslips in plastic six-well multidish plates and maintained at 37°C in a humidified incubator in an atmosphere of 95% air-5% CO2. Confluent cell cultures were rendered quiescent by deprivation and maintenance in serum-free medium for 36 hours before experimentation. The cells were identified as VSMCs by their typical "hill-and-valley" pattern at confluence and by testing positive for smooth muscle cell α-actin.

Measurement of [Ca2+]i in Single Vascular Smooth Muscle Cells

The quiescent cells grown on the glass coverslips were washed three times with Hanks' buffered saline solution of the following composition (mmol/L): NaCl 137, NaHCO3 4.2, Na2HPO4 3, KCl 5.4, KH2PO4 0.4, CaCl2 1.3, MgCl2 0.5, glucose 10, and HEPES 5, pH 7.4. The cells were loaded with fura-2, AM (4 μmol/L) that was dissolved in dimethyl sulfoxide with 0.02% pluronic acid and incubated at 37°C for 30 minutes. Thereafter, the loaded cells were washed three times with Hanks' buffer. This method allowed for examination of four cells per coverslip. The coverslip containing cells and rings was placed in a chamber and mounted on the stage of an inverted microscope equipped with epifluorescence with a ×40 oil immersion objective (Nikon). Fluorescence measurements were performed using double excitation wavelength measurements (345/380 nm) and a single-emission wavelength (510 nm) using a F12 D401 microfluorometer (Photon Technology International Inc.). Background fluorescence was determined for every cell studied, and this was subtracted before ratio measurements were obtained. Maximum and minimum fluorescent intensities were obtained for each cell by exposure to 10 μmol/L ionomycin and 3 mmol/L EGTA, respectively. [Ca2+]i was determined according to the formula of Grynkiewicz et al.1 with a binding constant (Kd) of Ca2+ to fura-2 of 224 nmol/L.

Protocols for Reagent Applications

Basal and insulin (70 μU/mL)-stimulated [Ca2+]i were measured in 20 to 30 cells per rat group. The maximum peak ratio recorded was considered the maximal response of the agonist. Responsiveness of the cells to insulin was greater than 96%. The effects of various modulators of calcium transport, including genistein, tyrphostin 23, calphostin C, staurosporine, thapsigargin, and diltiazem, on insulin-induced [Ca2+]i transients were also determined. The cells were preincubated for 5 to 10 minutes with 0.1 μmol/L of each of the agents before administration of insulin (70 μU/mL). Single cells were exposed to one agent only and were not reused for any further experiments. For each agent six to eight cells per rat group were studied.

Statistical Analyses

Data are presented as mean±SD unless otherwise stated. Statistical analyses were performed by ANOVA followed by Bonferroni's correction for multiple testing or Student's t-test as appropriate to compare results between groups. Differences were considered significant at a value of P<0.05.

Results

Blood pressure was significantly higher and body weight significantly lower in the SHR compared with the WKY and Wistar rats (P<0.01) (Table). Basal [Ca2+]i was determined from many cells (n=25 to 30 per group) derived from at least 20 rats per strain. In cells from 3-week-old rats there were no significant differences in basal [Ca2+]i (Table). Unstimulated [Ca2+]i was significantly higher in cells from 17-week-old SHR compared with age-matched WKY and Wistar cells (134±8 versus 98±12 and 99±10 nmol/L, SHR versus WKY and Wistar, P<0.01) (Table). Insulin caused a significant increase in [Ca2+]i in 17-week-old WKY, Wistar, and SHR VSMCs, but the peak [Ca2+]i was significantly higher in the SHR group (177±7 versus 130±14 and 132±16 nmol/L, SHR versus WKY and Wistar, P<0.01) (Table). Insulin significantly (P<0.05) increased [Ca2+]i, by 43±6 nmol/L in the SHR group, compared with 32±4 nmol/L in the WKY group and 33±3 nmol/L in the Wistar group. Maximal responses to insulin were achieved within 150 seconds, and baseline or plateau levels were obtained within 300 seconds. [Ca2+]i recovery after insulin administration was significantly delayed in the SHR cells (Fig 1).

The effects of various modulators of cell calcium on insulin-sensitive [Ca2+]i were determined in cells derived from 17-week-old SHR and WKY rats. Insulin-stimulated calcium responses in both groups were completely abolished by the tyrosine kinase inhibitors genistein and tyrphostin 23 (Figs 2 and 3). The voltage-dependent calcium channel blocker diltiazem and the protein kinase C inhibitors staurosporine and calphostin C significantly (P<0.01) reduced the effects of insulin in the SHR and WKY cells, but the inhibitory effect in the WKY cells was significantly greater (P<0.05) than that in the SHR cells (Figs 2 through 4). There were no significant differences in the effects between staurosporine and calphostin C (Figs 2 through 4). Thapsigargin, an inhibitor of reticular Ca2+-ATPase, increased [Ca2+]i from 227±20 nmol/L in the WKY cells and to 336±13 nmol/L in the SHR cells.

### Table

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Rat Strain</th>
<th>Weight, g</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Basal [Ca2+]i, nmol/L</th>
<th>Insulin-Stimulated [Ca2+]i, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Wistar</td>
<td>57.0±1.0</td>
<td>102±2.1</td>
<td>96±8</td>
<td>135±10</td>
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<td></td>
<td>WKY</td>
<td>74.4±0.9*</td>
<td>102±2.4</td>
<td>97±10</td>
<td>130±6</td>
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<tr>
<td></td>
<td>SHR</td>
<td>28.7±0.5</td>
<td>123±3.0*</td>
<td>110±6</td>
<td>140±8</td>
</tr>
<tr>
<td>17</td>
<td>Wistar</td>
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<td>109±2.9</td>
<td>99±10</td>
<td>132±16</td>
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<tr>
<td></td>
<td>WKY</td>
<td>477±3.3</td>
<td>121±2.3</td>
<td>98±12</td>
<td>130±14</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>328±2.0*</td>
<td>206±2.3*</td>
<td>134±8*</td>
<td>177±7*</td>
</tr>
</tbody>
</table>

VSMC indicates vascular smooth muscle cell; WKY, Wistar-Kyoto rats; and SHR, spontaneously hypertensive rats. *P<0.01 vs other groups of same age.
Fig 1. Line graph shows time course of \([Ca^{2+}]_i\) changes after insulin (70 \(\mu\)U/mL) stimulation. Maximal stimulated \([Ca^{2+}]_i\) was taken at 0 seconds, and recovery time to basal values was measured thereafter. Numbers in parentheses indicate cell number studied per group. SHR indicates spontaneously hypertensive rats; WKY, Wistar-Kyoto rats. Results are mean±SD. *P<.05 vs SHR basal \([Ca^{2+}]_i\); **P<.05 vs WKY basal \([Ca^{2+}]_i\).

nmol/L in the SHR cells. The thapsigargin-induced \([Ca^{2+}]_i\) increase was significantly higher (P<.01) in the SHR VSMCs (202±28 nmol/L) compared with the WKY VSMCs (130±15 nmol/L) (P<.01). In the presence of thapsigargin insulin failed to increase \([Ca^{2+}]_i\) above the concentration induced by thapsigargin alone. The maximal \([Ca^{2+}]_i\), response to thapsigargin in the presence of insulin was significantly (P<.05) greater in the SHR cells compared with the WKY cells (Figs 1 and 2). However, the change in \([Ca^{2+}]_i\) from basal, induced by thapsigargin in the presence of insulin, was not significantly different between WKY and SHR cells (98±6 nmol/L versus 134±23 nmol/L, respectively).

**Discussion**

This study demonstrates that physiological concentrations of insulin increase \([Ca^{2+}]_i\) in quiescent primary cultured unpassaged rat VSMCs and that responses are significantly enhanced in cells from adult SHR. Two normotensive strains were studied to avoid the confounding effect of possible genetic heterogeneity of WKY rats. \([Ca^{2+}]_i\) responses were similar in both WKY and Wistar VSMCs. In VSMCs from 3-week-old animals the insulin-mediated \([Ca^{2+}]_i\) increase was similar in the hypertensive and normotensive groups, whereas in cells from 17-week-old SHR, when hypertension was fully established, basal and insulin-stimulated \([Ca^{2+}]_i\) were significantly increased. These data suggest that altered cellular responses to insulin may not be a primary defect but rather a secondary event in hypertension. Other investigators have also documented differences in insulin-sensitive \([Ca^{2+}]_i\) responses between WKY and SHR cells. Kuriyama et al demonstrated a blunted response of insulin-induced \(Ca^{2+}\) uptake and \([Ca^{2+}]_i\) in SHR VSMCs and attributed this to possible downregulation of the insulin receptor. These results differ from our data and may be due to the fact that serially passaged cultured VSMCs (five to eight passages) obtained from thoracic aortas of 12-week-old rats were examined in the study of Kuriyama et al, whereas

Fig 2. Bar graph shows effects of various modulators of calcium transport on basal and insulin-induced \([Ca^{2+}]_i\) in primary cultured vascular smooth muscle cells (VSMCs) derived from 17-week-old Wistar-Kyoto (WKY) rats. Six to eight cells were studied for each agent. Tyr indicates tyrphostin; I, insulin; Gen, genistein; Dil, diltiazem; Stau, staurosporine; Cal, calphostin C; and Thap, thapsigargin. Results are mean±SD. *P<.01 vs other groups.

Fig 3. Bar graph shows effects of various modulators of calcium transport on basal and insulin-induced \([Ca^{2+}]_i\) in primary cultured vascular smooth muscle cells (VSMCs) derived from 17-week-old spontaneously hypertensive rats (SHR). Six to eight cells were studied for each agent. Tyr indicates tyrphostin; I, insulin; Gen, genistein; Dil, diltiazem; Stau, staurosporine; Cal, calphostin C; and Thap, thapsigargin. Results are mean±SD. *P<.01 vs other groups.

Fig 4. Bar graph shows effect of \(Ca^{2+}\) transport modulators on insulin-induced \([Ca^{2+}]_i\) in vascular smooth muscle cells (VSMCs) of Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR). Data are presented as the \([Ca^{2+}]_i\) difference between \([Ca^{2+}]_i\), mediated by insulin and \([Ca^{2+}]_i\) mediated by insulin plus \(Ca^{2+}\) inhibitor. In indicates insulin; Tyr, tyrphostin; Gen, genistein; Dil, diltiazem; Stau, staurosporine; and Cal, calphostin C. Results are expressed as mean±SD. *P<.05 vs WKY group.
we studied only primary unpassaged cells derived from resistance vessels of the mesenteric vasculature. Implications of increased VSMC [Ca\(^{2+}\)]\(,\) induced by insulin remain unclear. Although some studies have suggested that insulin has a vasodilator effect, others have implicated a vasoconstrictor role of insulin. Insulin attenuates agonist-induced [Ca\(^{2+}\)], transients in VSMCs and blunts vasoconstriction by various agonists in rat mesenteric arterioles. Most studies have failed to demonstrate insulin-mediated vasoconstriction. This may be partially due to the fact that although insulin raises [Ca\(^{2+}\)], the rise may not be high enough to induce vasoconstriction, especially if it is not accompanied by an increase in myofilament sensitivity to calcium or to activation of other signal transduction pathways that act with calcium to cause vasoconstriction. We propose that insulin-sensitive [Ca\(^{2+}\)], transients may not be directly involved in vasoconstriction but may rather be an integrative step in eliciting responses to other vasoactive agents that are important in modulating vascular tone and contractility. They may also relate to effects of insulin on VSMC growth, which could be independent of vasoconstrictor effects. Direct insulin-induced [Ca\(^{2+}\)], changes may in fact operate in a direction opposite to other insulin-induced effects on [Ca\(^{2+}\)], namely, interactions with agonist-evoked [Ca\(^{2+}\)], transients. We have recently demonstrated that insulin attenuates agonist-evoked calcium transients in rat VSMCs and suggested, like others, that insulin may confer some protection against exaggerated vasoconstriction. These contrasting actions of insulin on [Ca\(^{2+}\)], might be attributed to calcium mobilization from different subcellular pools or differential compartmentalization within the cell. By examining the action of insulin on [Ca\(^{2+}\)], alone and its interaction with vasoconstrictors, apparently opposing calcium modulator effects of insulin may be dissected. These may have distinct physiopathological significance. We speculate that the direct effect may be related to the growth-promoting action of insulin on VSMCs, whereas the interactive effect may result in its vasodilator or antivasoconstrictor action, which remains to be demonstrated.

Mechanisms by which insulin increases VSMC [Ca\(^{2+}\)], are unclear. Insulin may influence [Ca\(^{2+}\)], by influx from the extracellular compartment, mobilization from cytoplasmic stores, or cytoplasmic disposal of calcium. The present study further clarifies some possible pathways and suggests mechanisms by which insulin-evoked [Ca\(^{2+}\)], responses may be increased in SHR VSMCs. Genistein and tyrphostin 23, which are tyrosine kinase inhibitors, completely abolished the insulin-sensitive [Ca\(^{2+}\)], effect. Because insulin receptors are known to possess tyrosine kinase activity, these responses are not unexpected and confirm that in VSMCs, as in other cell types, insulin effects are mediated via tyrosine kinase activation. Calphostin C and staurosporine reduced insulin-mediated VSMC [Ca\(^{2+}\)], transients but did not abolish them. Staurosporine is a relatively nonspecific protein kinase C inhibitor, whereas calphostin C is a highly selective protein kinase C inhibitor. These data suggest that changes in intracellular [Ca\(^{2+}\)], may be modulated in part by protein kinase C-mediated mechanisms. These results confirm previously published data from VSMCs derived from Sprague-Dawley and WKY rats as well as from human mesangial cells. Diltiazem, one of many voltage-dependent Ca\(^{2+}\) channel antagonists, significantly blocked Ca\(^{2+}\) responses to insulin. This probably occurs via inhibition of L-type Ca\(^{2+}\) channels, which may be insulin sensitive. In cultured aortic cells insulin-evoked calcium uptake has also been found to be inhibited by Ca\(^{2+}\) antagonists such as diltiazem. Other mechanisms whereby diltiazem could affect insulin-mediated calcium mobilization could be by changes in basal [Ca\(^{2+}\)], or calcium stores, indirectly through its effect on calcium channels.

Most studies investigating mechanisms of insulin-related [Ca\(^{2+}\)], signaling have been performed in cells from normotensive animals. As far as we know the present study demonstrates for the first time differences in insulin-sensitive Ca\(^{2+}\) transport between primary cultured, unpassaged VSMCs derived from normotensive and hypertensive rats. This study shows that the inhibitory effects of diltiazem and protein kinase C inhibitors on insulin-induced [Ca\(^{2+}\)], transients are significantly reduced in SHR cells compared with WKY cells. From these data it could be suggested that in SHR VSMCs L-type Ca\(^{2+}\) channel-mediated and protein kinase C-mediated calcium transport may be partially resistant to blockers, which in turn may be due to underlying alterations in protein kinase C activity, to changes in Ca\(^{2+}\) channels, or to impaired antagonist affinity. Altered protein kinase C and Ca\(^{2+}\) channel activation have been demonstrated in SHR cells. Exaggerated [Ca\(^{2+}\)], responses to insulin in SHR could also be due to delayed [Ca\(^{2+}\)], recovery after insulin stimulation. WKY cells returned to baseline very soon after agonist administration, whereas SHR cells did not reach basal values within 5 minutes after insulin application. Similar trends have been shown in cells from insulin-resistant Zucker obese hypertensive rats when stimulated with angiotensin II.

Insulin could also modulate [Ca\(^{2+}\)], by influencing Ca\(^{2+}\) release from cytoplasmic stores. In the present study we investigated the association between insulin and intracellular calcium mobilization with thapsigargin. Thapsigargin inhibits reticular Ca\(^{2+}\)-ATPase and elevates [Ca\(^{2+}\)], by preventing reentry of calcium into the endoplasmic reticular storage sites. Thapsigargin significantly increased VSMC [Ca\(^{2+}\)]. In the presence of this inhibitor, insulin failed to elevate [Ca\(^{2+}\)], further, suggesting that in addition to increased influx, insulin-induced [Ca\(^{2+}\)], responses may be influenced by intracellular calcium mobilization. However, it should be stressed that because thapsigargin itself increases [Ca\(^{2+}\)], insulin may not be able to further raise [Ca\(^{2+}\)]. Thapsigargin-induced [Ca\(^{2+}\)], increase was significantly greater in SHR VSMCs than in WKY cells. When cells were exposed to thapsigargin and insulin, the maximum increase was not significantly different between WKY and SHR VSMCs. These results suggest that insulin effects on calcium mobilization from sarcoplasmic stores that utilize Ca\(^{2+}\)-ATPase as the main Ca\(^{2+}\) transporter may not differ between WKY and SHR cells.

In conclusion, physiological concentrations of insulin induce [Ca\(^{2+}\)], increases in VSMCs probably via voltage-dependent calcium channels, intracellular calcium release, and protein kinase C-mediated mechanisms. The insulin effects on [Ca\(^{2+}\)], are increased in SHR VSMCs
Insulin, Ca\(^{2+}\) in Vascular Smooth Muscle in SHR


Insulin-induced Ca2+ transport is altered in vascular smooth muscle cells of spontaneously hypertensive rats.
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