Insulin Inhibits Serotonin-Induced Ca\textsuperscript{2+} Influx in Vascular Smooth Muscle

Andrew M. Kahn, MD; Julius C. Allen, PhD; Charles L. Seidel, PhD; Tom Song, BS

Background Insulin in physiological concentrations attenuates the agonist-induced intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) transient and inhibits contraction in individual nonproliferated cultured canine femoral artery vascular smooth muscle cells (VSMCs). In the present study, we wished to define the effects of insulin on individual components of Ca\textsuperscript{2+} transport in vascular smooth muscle.

Methods and Results Insulin (40 \textmu M) attenuated the 5-hydroxytryptamine (5-HT, serotonin; 10^{-5} mol/L)-induced [Ca\textsuperscript{2+}] transient (measured by fura 2 fluorescence) in primary confluent canine femoral artery VSMCs in the presence of extracellular Ca\textsuperscript{2+}. In Ca\textsuperscript{2+}-free media, the 5-HT-induced [Ca\textsuperscript{2+}] transient was reduced by 42% and was not affected by insulin. This finding suggested that insulin inhibits 3-HT-induced Ca\textsuperscript{2+} influx but does not affect sarcolemmal Ca\textsuperscript{2+} efflux or Ca\textsuperscript{2+} release from intracellular stores. In support of those conclusions, we found that insulin inhibited the 5-HT-induced component of Mn\textsuperscript{2+} (a Ca\textsuperscript{2+} surrogate) influx (measured by fura 2 fluorescence quenching at the Ca\textsuperscript{2+} isosbestic excitation wavelength). In addition, 5-HT stimulated the rates of Ca\textsuperscript{2+} influx from intact cells (a measure of sarcolemmal Ca\textsuperscript{2+} efflux) and from saponin-permeabilized cells (a measure of Ca\textsuperscript{2+} release from intracellular stores), but insulin did not affect these rates of Ca\textsuperscript{2+} influx.

Conclusions We conclude that a physiological insulin concentration attenuates the 5-HT-induced [Ca\textsuperscript{2+}] transient in confluent primary cultured canine femoral artery VSMCs by inhibiting the 5-HT-induced component of Ca\textsuperscript{2+} influx but not by affecting sarcolemmal Ca\textsuperscript{2+} efflux or Ca\textsuperscript{2+} release from intracellular stores. (Circulation. 1994;90:384-390)

Key Words • calcium • hypertension • insulin

Arterial hypertension is associated with non-insulin-dependent diabetes and obesity. Patients with these conditions, as well as some groups of patients with essential hypertension, demonstrate resistance to insulin-induced glucose disposal.\textsuperscript{1,4} It has also been reported recently that physiological concentrations of insulin cause vasodilation in normal men\textsuperscript{1,4} and inhibit agonist-induced vasoconstriction in vivo\textsuperscript{2} as well as agonist-induced contraction of individual vascular smooth muscle cells (VSMCs) in vitro.\textsuperscript{6} Resistance to insulin-induced vasodilation is associated with resistance to insulin-induced glucose disposal in patients with non-insulin-dependent diabetes,\textsuperscript{4} obesity,\textsuperscript{3} and essential hypertension.\textsuperscript{7} Resistance to the normal effect of insulin to inhibit agonist-induced contraction of vascular smooth muscle could contribute, therefore, to the pathogenesis of hypertension in these disorders.

Several laboratories have demonstrated that insulin attenuates the agonist-induced intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) transients in cultured rat VSMCs and affects various sarcolemmal and sarcoplasmic reticular Ca\textsuperscript{2+} transport systems.\textsuperscript{8-13} The physiological relevance of these findings remains uncertain because supraphysiological insulin concentrations were used in most of these experiments, which may not reflect the action of physiological concentrations of the hormone.\textsuperscript{14} We have shown previously that a physiological insulin concentration (40 \textmu M/mL) inhibits both the agonist-induced contraction and [Ca\textsuperscript{2+}]\textsubscript{i} transient in individual nonproliferated cultured canine femoral artery VSMCs.\textsuperscript{6} We wished to examine the mechanism by which insulin affects this [Ca\textsuperscript{2+}] transient. Because the yield of these cells is low, it is difficult to perform the \textsuperscript{45}Ca\textsuperscript{2+} flux studies necessary to achieve this goal. Therefore, populations of confluent primary cultured canine femoral artery VSMCs were used in the present study to define the mechanism by which a physiological concentration of insulin inhibits the [Ca\textsuperscript{2+}] transient. It is demonstrated that insulin inhibits Ca\textsuperscript{2+} influx induced by 5-hydroxytryptamine (5-HT, serotonin) but does not affect sarcolemmal Ca\textsuperscript{2+} efflux or Ca\textsuperscript{2+} release from intracellular stores.

Methods

Cell Culture

Adult mongrel dogs of either sex were killed with intravenous pentobarbital sodium, and the femoral arteries were dissected free. Primary cultures of VSMCs were prepared as previously described.\textsuperscript{15} In brief, the media of the arteries were minced and incubated at 37°C in a solution containing elastase (type V, Sigma Chemical Co) and collagenase (type I, Worthington Biochemicals). After 2 hours, the enzyme solution was discarded and replaced with fresh solution, and the tissue was incubated for an additional 2 hours. The dispersed cells were pelleted and suspended in complete Dulbecco's modified Eagle's medium (DMEM; Gibco), which contained 10% fetal calf serum (FCS; Cyclone), 1% glutamine, and 1% PS (10 000 U/mL penicillin and 10 mg/mL streptomycin; Sigma). To grow cells on coverslips, the cell suspension was adjusted to 1.7 \times 10^4 cells/mL, and 0.3 mL was placed on the surface of 10x22-mm glass coverslips. To grow cells on the bottom of culture dishes, the suspension of dispersed cells was adjusted to 2 \times 10^5 cells/mL, and 1 mL was placed in 35-mm plastic dishes (Falcon). The coverslips and dishes were placed in a humidified tissue culture incubator maintained at 37°C and equili-
brated with 5% CO₂-95% air. After 72 hours and every 72 hours thereafter, the media were replaced with 1 mL of fresh complete DMEM. The cells that were plated on coverslips or on culture dishes reached confluence between the 10th and 15th day. The identity of the cultured cells as smooth muscle cells was confirmed as previously described by the "hill and valley" pattern of cell growth and by an actin-to-mycosin heavy chain ratio characteristic of intact vascular smooth muscle.16 Only confluent primary cultures of cells were used in these studies.

**Fluorescence Measurements of Ca²⁺ Transport**

A coverslip with cells was placed in a quartz cuvette inside a fluorescent spectrophotometer (Perkin-Elmer LS-3B) such that the coverslip was anchored at its bottom and top and sat at a 45° angle to the excitation beam. The cuvette (2 mL), which was held in a thermostated holder, was superfused with solution at 37°C containing (in millimoles per liter) 140 NaCl, 4 KCl, 1.8 CaCl₂, 0.8 MgCl₂, 5 glucose, 10 HEPES-Tris [pH 7.4], plus 0.1% bovine serum albumin (physiological salt solution (PSS)) or Ca²⁺-free PSS that lacked added Ca²⁺ and contained 1 mmol/L EGTA. A peristaltic perfusion pump delivered solution at 3 mL/min into the bottom of the cuvette via a polyethylene tube, which ran on the opposite side of the coverslip from the excitation light and pumped solution at 3 mL/min from the top of the cuvette. The half-time for the turnover of solution in the cuvette was 0.46 minutes. The solutions perfused were determined at 37°C. Before the experiment, the cells were exposed to Ca²⁺-sensitive dye, they were excited at 340 nm through a 10-nm slit width, the emitted light at 510 nm was monitored through a 10-nm slit width, and the intensity was blanked to zero units. The excitation beam was changed to 380 nm, and the emission signal at 510 nm was arbitrarily set to 150 units. This value was subtracted from all subsequent values obtained after cells had been loaded with dye to obtain fluorescence data from the dye itself without the contribution of autofluorescence from the cells or other components in the cuvette. The cells were loaded in the cuvette for 45 minutes with 2.4 μmol/L furam 2-AM (Molecular Probes) that had been sonicated for 20 seconds in DMEM with 0.1% bovine serum albumin. The cuvette was perfused again at 1 mL/min with PSS with or without bovine insulin (40 μU/mL) for 20 minutes. The cuvette was then perfused at 3 mL/min with PSS or Ca²⁺-free PSS with or without insulin, and baseline fluorescence emissions were obtained by rapidly alternating the excitation wavelength between 340 and 380 nm and recording the 510-nm emission intensity. 10⁻³ mol/L 5-HT was added to the perfusion solution, and fluorescence measurements continued for 20 minutes. [Ca²⁺]ᵢ values were calculated from the fluorescence ratio recordings using the following formula:

\[ [Ca^{2+}]_i = K_d (R_{min} - R_{max} - R) / K_p \]

where \( R \) is the ratio of the experimental fluorescence intensity at 510 nm to the fluorescence intensity at 340 nm, and \( K_d \) and \( K_p \) are the dissociation and association constants, respectively. The values for the dissociation and association constants were determined by fitting the data to the equation \( [Ca^{2+}]_i = K_d (R_{min} - R_{max} - R) / K_p \) using a nonlinear least-squares regression analysis.

To assess the rate of Mn²⁺ influx (a putative marker for Ca²⁺ influx), coverslips were loaded with furam 2 and preincubated with or without insulin, as described above, and then superfused with nominally Ca²⁺-free HEPES PSS. Cells were excited at 355 nm, which is the Ca²⁺ isosbestic point for intracellular furam 2 in these cells using this instrument, as determined in preliminary experiments. The emission at 510 nm was continuously recorded. When a stable value was obtained, 0.5 mmol/L MnCl₂ was added to the superfusion solution, followed by the same solution with or without 10⁻³ mol/L 5-HT. The rate of fluorescence quenching was taken as an estimate of Mn²⁺ influx, as previously described.18-20

To standardize this rate from coverslip to coverslip, the superfusion solution was changed again to nominally Ca²⁺-free HEPES PSS containing 5 mmol/L MnCl₂ plus 2.5 μmol/L ionomycin. This ionophore rapidly allowed Mn²⁺ influx and quenched the fluorescence to a stable basal value. The difference between this value and the initial stable value before exposing the cells to Mn²⁺ was taken as 100 arbitrary fluorescence units. After the superfusion solution was changed to one containing 0 or 10⁻⁵ mol/L 5-HT, the initial rate of fluorescence decline was calculated and expressed in terms of arbitrary fluorescence units per minute.

**Ca²⁺ Efflux**

Cells were loaded with ⁴⁰Ca₂⁺ (5 μCi/mL) in PSS at 37°C for 3 hours. The cells were preincubated with or without 40 μU/mL insulin for the last 20 minutes and with and without 20 μg/mL saponin for the last 5 minutes. They were washed 10 times at room temperature with 1 mL of unlabeled PSS (approximately 20 seconds in duration) and then incubated with 1 mL of PSS at 37°C in the continued presence or absence of insulin and in the presence or absence of 10⁻³ mol/L 5-HT. Every 30 seconds the incubation solution was replaced with 1 mL of fresh solution. After the fifth exchange of incubation solution had been removed, the cells were lysed in 1 mL of 0.5 N NaOH for 30 minutes. The amount of ⁴⁰Ca₂⁺ that had left the cells in each of the 30-second intervals and the amount of ⁴⁰Ca₂⁺ that remained associated with the cells were determined by liquid scintillation spectroscopy. The data were fitted to the equation \( CPM = CPM_p e^{-kt} \), where \( CPM \), \( CPM_p \) and \( CPM_p e^{-kt} \) are the amounts of isotope associated with the cells at times zero and \( r \) after initiation of efflux, respectively, and \( k \) is a constant. Each experiment was performed in triplicate. The data fit the above equation with a regression coefficient of 0.9 or greater in every experiment. The value for \( k \) was taken to represent the first-order rate constant for ⁴⁰Ca₂⁺ efflux.

⁴⁰Ca (25 μCi/μg) was obtained from New England Nuclear, and bovine insulin, 5-HT, ionomycin, and saponin were obtained from Sigma. Statistical analysis was performed on paired data by use of Student’s test (Fig 4) and ANOVA with multiple comparisons using the Newman-Keuls test (Tables 1 and 2, Fig 1). Statistical significance was taken as a value of \( P < 0.05 \).

**Results**

To determine whether a physiological concentration of insulin attenuated the 5-HT-induced [Ca²⁺]ᵢ transient in primary confluent cultures of canine femoral artery VSMCs, [Ca²⁺]ᵢ was monitored in cells preincubated with or without 40 μU/mL insulin for 20 minutes before stimulation with 10⁻³ mol/L 5-HT. Insulin did not affect the basal concentration of [Ca²⁺]ᵢ, which averaged 82±5 and 79±7 nmol/L in the presence and absence of insulin, respectively (n=6 coverslips without and n=6 coverslips with insulin, P=NS). As shown in the upper panel of Fig 1, 5-HT increased [Ca²⁺]ᵢ to 4.3±0.4 times the basal concentration at the 30-second peak in the absence of insulin, but to only 3.0±0.5 times the basal concentration in the presence of insulin (P<.05). Thus, a physiological concentration of insulin attenuated the 5-HT-induced [Ca²⁺]ᵢ transient in these cells. To determine whether 5-HT-stimulated Ca²⁺ influx was in part responsible for the [Ca²⁺]ᵢ transient, and if so, whether insulin inhibited this component of Ca²⁺ transport, [Ca²⁺]ᵢ was monitored in cells preincubated for 20 minutes with or without 40 μU/mL insulin; however, immediately before addition of 10⁻⁵ mol/L 5-HT, the perfusion solution was changed to one containing no added Ca²⁺ plus 1 mmol/L EGTA, which did not acutely affect [Ca²⁺]ᵢ (data not shown). As shown in
the lower panel of Fig 1, 5-HT increased [Ca\(^{2+}\)] to only 2.5±0.3 times the basal concentration (P<.05 versus Ca\(^{2+}\)-replete perfusion solution), and insulin had no detectable effect on this value (2.2±0.3, P=NS). Taken together, the data in Fig 1 demonstrate that 40 μU/mL insulin attenuated the 5-HT-induced [Ca\(^{2+}\)] transient in these cells and that 5-HT-stimulated Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular stores contribute to the 5-HT-induced [Ca\(^{2+}\)] transient. Since we found no significant inhibition of the 5-HT-induced [Ca\(^{2+}\)] transient by insulin in Ca\(^{2+}\)-free media, the data in Fig 1 also suggest that the inhibition of the [Ca\(^{2+}\)] transient by insulin in Ca\(^{2+}\)-replete media occurs via inhibition of 5-HT–stimulated Ca\(^{2+}\) influx, not by stimulation of sarcolemmal Ca\(^{2+}\) efflux or inhibition of Ca\(^{2+}\) release from intracellular stores.

To confirm that insulin inhibited the 5-HT–induced component of Ca\(^{2+}\) influx, we measured the effect of insulin on 5-HT–stimulated Mn\(^{2+}\) influx in these cells. Mn\(^{2+}\) influx can be used as a surrogate for Ca\(^{2+}\) influx via Ca\(^{2+}\) channels in a variety of cell types, including VSMCs. As has been reported by others, we took advantage of the fact that the fluorescence of intracellular fura 2 is quenched by intracellular Mn\(^{2+}\) at excitation wavelengths including the Ca\(^{2+}\) isosbestic wavelength of fura 2. Because changes in [Ca\(^{2+}\)], do not affect the emission of fura 2 when excited at its isosbestic wavelength, experimental maneuvers that increase intracellular Mn\(^{2+}\) will quench fura 2 fluorescence, and this effect will be insensitive to changes in [Ca\(^{2+}\)]. Cells grown on coverslips were loaded with fura 2 and superfused with PSS that was nominally Ca\(^{2+}\)-free. When 0.5 mmol/L Mn\(^{2+}\) was added to the superfusion solution, the 510-nm emission of fura 2, which was excited at 355 nm, was quenched as shown in Fig 2. This result can be explained by Mn\(^{2+}\) influx into the cell. As also shown in Fig 2, when 10^-5 mol/L 5-HT was added to the superfusion solution, the rate of fluorescence quenching increased. This finding is consistent with previous reports showing that 5-HT increases the influx of Ca\(^{2+}\) in vascular smooth muscle. Ionomycin, which is also a Mn\(^{2+}\) ionophore, further accelerated the rate of fura 2 quenching.

**Fig 1.** Graphs showing effect of insulin on 5-hydroxytryptamine (5-HT)–induced [Ca\(^{2+}\)], transient in vascular smooth muscle cells. Cells grown on coverslips were preloaded with fura 2 and preincubated for 20 minutes with or without 40 μU/mL insulin. [Ca\(^{2+}\)] was measured by fluorescence spectroscopy. Top, Cells were superfused with physiological salt solution (PSS) and then exposed to 10^-5 mol/L 5-HT. Bottom, Cells were superfused with PSS, then superfused with Ca\(^{2+}\)-free PSS, and immediately exposed to 10^-5 mol/L 5-HT. Data represent the ratio of [Ca\(^{2+}\)], divided by the basal [Ca\(^{2+}\)], which was determined immediately before exposing the cells to 5-HT. Data are the means±SE from seven experiments performed under each set of conditions. *P<.05 versus insulin, Ca\(^{2+}\)-free, and Ca\(^{2+}\)-free plus insulin.
**Fig 1.** Graph showing effect of 5-hydroxytryptamine (5-HT) and insulin on Mn⁺² influx in vascular smooth muscle cells. Cells were assayed by \((\Delta F/\Delta t)_m\) with fura 2 and preincubated in physiological salt solution (PSS) with or without 40 μU/mL insulin for 20 minutes. The cells were superfused with PSS without added Ca²⁺ and were then preincubated with or without 10⁻⁵ mol/L 5-HT. The rate of fluorescence quenching was determined. Data represent the means±SE for four coverslips in each group. *P<.05 vs control; †P<.05 vs 5-HT.

**Fig 2.** Graph showing effect of 5-hydroxytryptamine (5-HT) on Mn⁺² influx in vascular smooth muscle cells. Cells on a coverslip were loaded with fura 2 and excited at 355 nm, and the fluorescence emission at 506 nm was monitored. The cells were superfused with physiological salt solution in the nominal absence of Ca²⁺. At the indicated times, 0.5 mmol/L Mn⁺², 10⁻⁵ mol/L 5-HT, and 2.5 μmol/L ionomycin were added to the superfusion solution. Tracing is from a representative experiment.

**Table 1.** Effects of 5-HT and Insulin on Mn⁺² Influx

<table>
<thead>
<tr>
<th>Group</th>
<th>Fura 2 Fluorescence Quench Rate, AU/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.3±0.5</td>
</tr>
<tr>
<td>5-HT</td>
<td>9.1±0.5*</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>5-HT+insulin</td>
<td>6.2±0.2†</td>
</tr>
</tbody>
</table>

Table 1 shows the effect 10⁻⁵ mol/L 5-HT and 40 μU/mL insulin on the rate of fura 2 quenching in cells bathed in 0.5 mmol/L Mn⁺². 5-HT increased the rate of quenching three-fold. Preincubation with insulin did not significantly affect the basal rate of quenching, but insulin inhibited fura 2 quenching of 5-HT–treated cells by 32% and inhibited the 5-HT–induced component of fura 2 quenching by 40%. The data in Table 1 are consistent with the notion that insulin inhibits the 5-HT–induced component of Ca²⁺ influx.

To confirm that insulin does not affect sarcolemmal Ca²⁺ efflux, cells were loaded with \(^{45}\text{Ca}^2⁺\) for 3 hours and preincubated with or without 40 μU/mL insulin for the last 20 minutes. Insulin did not affect the amount of \(^{45}\text{Ca}^2⁺\) preloaded into the cells (data not shown). \(^{45}\text{Ca}^2⁺\) efflux was assayed in the presence and absence of 10⁻⁵ mol/L 5-HT and insulin for 20 minutes. As shown in Fig 3 and Table 2, 5-HT stimulated \(^{45}\text{Ca}^2⁺\) efflux, but insulin had no effect on \(^{45}\text{Ca}^2⁺\) efflux in the presence or absence of 5-HT. The stimulation of \(^{45}\text{Ca}^2⁺\) efflux by 5-HT was expected because 5-HT stimulates \(^{45}\text{Ca}^2⁺\) release from intracellular stores (Fig 1, bottom). The finding that insulin had no effect on \(^{45}\text{Ca}^2⁺\) efflux indicates that insulin does not stimulate sarcolemmal \(^{45}\text{Ca}^2⁺\) efflux and thus supports the data in Fig 1.

To determine the effects of insulin on the release of Ca²⁺ from intracellular stores, we studied saponin-skinned cells. To verify that saponin effectively increased sarcolemmal Ca²⁺ permeability in these cells, the uptake of \(^{45}\text{Ca}^2⁺\) was assayed by cells that had been preincubated with or without 20 μg/mL saponin for 5 minutes. As shown in Fig 4, saponin increased \(^{45}\text{Ca}^2⁺\) uptake, indicating that this agent increased sarcolemmal Ca²⁺ permeability or increased Ca²⁺ binding by the sarcolemma and/or intracellular sites. When cells were preincubated with 20 μg/mL saponin for 5 minutes, the rate constant for \(^{45}\text{Ca}^2⁺\) efflux was increased (Figs 3 and 5, Table 2). Thus, the use of saponin in these studies increases sarcolemmal permeability to Ca²⁺ because if saponin merely increased the binding of Ca²⁺ by cell components, this agent would decrease, not increase, Ca²⁺ efflux.

**Table 2.** Effect of 5-HT and Insulin on \(^{45}\text{Ca}^2⁺\) Efflux from Vascular Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>No Saponin</th>
<th>Saponin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.11±0.01</td>
<td>0.18±0.01</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.13±0.02‡</td>
<td>0.18±0.02‡</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.25±0.01*</td>
<td>0.27±0.02*</td>
</tr>
<tr>
<td>5-HT+insulin</td>
<td>0.24±0.02‡</td>
<td>0.25±0.03†</td>
</tr>
</tbody>
</table>

5-HT indicates 5-hydroxytryptamine. Data are from Figs 3 and 5.

*P<.05 vs the same conditions without 5-HT; †P=NS vs the same conditions without insulin.
To confirm that insulin does not affect release of Ca\(^{2+}\) from intracellular stores, 45Ca\(^{2+}\) efflux was measured from preloaded cells that had been preincubated with or without 40 \(\mu\)U/mL insulin for 20 minutes and whose sarcolemma had been permeabilized to Ca\(^{2+}\) with saponin, as described above. Insulin did not affect the amount of 45Ca\(^{2+}\) preloaded into these permeabilized cells (data not shown). Efflux was measured in the presence or absence of 10\(^{-2}\) mol/L 5-HT with or without the continued presence of insulin. As shown in Fig 5 and Table 2, 5-HT increased the rate of 45Ca\(^{2+}\) efflux from these cells. Thus, the binding of 5-HT to its sarcolemmal receptor(s) and the signal transduction system that mediates the subsequent release of Ca\(^{2+}\) from intracellular stores remain intact after saponin treatment. As also shown in Fig 5 and Table 2, insulin had no effect on the rate of 45Ca\(^{2+}\) efflux from permeabilized cells in the absence or presence of 5-HT. These findings suggest that insulin does not inhibit 5-HT-induced Ca\(^{2+}\) release from the intracellular stores and thus also support the data in Fig 1.

**Discussion**

Several investigators have reported that physiological concentrations of insulin attenuate contraction of vascular smooth muscle in vivo and in vitro.\(^3\)-\(^6\) We have shown previously that 40 \(\mu\)U/mL insulin inhibits the 5-HT-induced contraction and [Ca\(^{2+}\)], transient in individual canine femoral artery VSMCs cultured in 0.5% FCS.\(^6\) Cells cultured under these conditions do not proliferate, and they maintain the contractile phenotype.\(^6\) We wished to investigate the mechanism by which insulin inhibited the [Ca\(^{2+}\)], transient. Because the yield of these cells is low, it is difficult to perform 45Ca\(^{2+}\) flux studies with this preparation. Thus, in the present study, canine femoral artery VSMCs that were cultured in 10% FCS were used as a model for the nonproliferated cells. These cells grow to confluence, which makes them more suitable than unproliferated cells for isotope flux studies.

In the present studies we show that confluent primary cultures of canine femoral artery VSMCs demonstrate a 5-HT–induced [Ca\(^{2+}\)], transient that is attenuated by a physiological concentration of insulin (40 \(\mu\)U/mL). These findings are identical to what we have observed in individual nonproliferated cells.\(^6\) We have shown that this attenuation of the [Ca\(^{2+}\)], transient by insulin can be explained by attenuation of the 5-HT–induced component of Ca\(^{2+}\) influx, but not by stimulation of sarcolemmal Ca\(^{2+}\) efflux or inhibition of Ca\(^{2+}\) release from intracellular stores. Each of these conclusions was supported individually by two different types of experiments. Fig 1 shows that 40 \(\mu\)U/mL insulin inhibits the 5-HT–induced [Ca\(^{2+}\)], transient in the presence but not absence of extracellular Ca\(^{2+}\). One conclusion from this experiment is that insulin inhibits 5-HT–induced Ca\(^{2+}\) influx. This conclusion was also supported by the finding that 40 \(\mu\)U/mL insulin inhibited the 5-HT–induced component of Mn\(^{2+}\) influx. While we have not shown in these studies that Ca\(^{2+}\) influx and Mn\(^{2+}\) influx occur by the same mechanism(s) in these cells, other studies of various cell types, including VSMCs, have shown that Ca\(^{2+}\) and Mn\(^{2+}\) share, at least in part, common sarcolemmal influx pathways.\(^18\)-\(^20\) We assume that the effects of 40 \(\mu\)U/mL insulin that we observed in these cells were mediated by insulin receptors, but the possibility exists that they were mediated, at least in part, by insulolinlike growth factor I or growth factor II receptors.

Two other conclusions that can be drawn from Fig 1 are that 40 \(\mu\)U/mL insulin neither (1) stimulates sarcolemmal Ca\(^{2+}\) influx nor (2) inhibits Ca\(^{2+}\) release from intracellular stores in 5-HT–stimulated cells. These conclusions seem warranted because, if they were not true, insulin would have attenuated the 5-HT–induced [Ca\(^{2+}\)], transient in the absence of extracellular Ca\(^{2+}\), and this was not observed. It should be pointed out that the data in Fig 1 cannot rule out the possibilities that, in the absence of extracellular Ca\(^{2+}\), insulin simultaneously stimulates or simultaneously inhibits both sarcolemmal Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular stores, such that insulin causes no change in the 5-HT–induced [Ca\(^{2+}\)], transient. These
possibilities seem unlikely, and the conclusions drawn from Fig 1 are further supported based on the data in Figs 3 and 5 and Table 2, which show that 40 μU/mL insulin does not affect 45Ca2+ efflux by the sarcolemma or affect 4Ca2+ release from intracellular stores in cells exposed to 5-HT.

Previous studies have examined the effects of insulin on Ca2+ transport in VSMCs from rats.8-10 Standley et al11 reported that 1 and 100 mU/mL insulin or zero extracellular Ca2+ lowered the peak of the arginine vasopressin (AVP)–induced [Ca2+]i transient in a7r5 cells. Insulin (100 mU/mL) inhibited AVP-stimulated and depolarization-induced inward currents (presumably Ca2+ currents) in these cells. They concluded that insulin inhibits receptor-operated and voltage-operated Ca2+ channels. The present studies are in accord with those of Standley et al and extend those observations by showing that a physiological concentration of insulin inhibits the agonist-induced [Ca2+]i transient and the agonist-induced component of Ca2+ influx. Zemel and coworkers13 reported that 0.1 mU/mL insulin enhanced relaxation of phrenyplehrine-contracted rat aortic strips and increased the rate of vanadate-sensitive, external Na+-insensitive 4Ca2+ efflux from unstimulated strips. In separate studies they demonstrated that 10−4 mol/L insulin (1.3 mU/mL) stimulated the rate of decline of the AVP- or angiotensin II–induced [Ca2+]i transient in a7r5 cells.10 Their studies suggest that insulin stimulates the sarcolemmal ATP-dependent Ca2+ pump in vascular smooth muscle, resulting in an attenuated agonist-stimulated [Ca2+]i transient. Tuck and coworkers (Saito et al8 and Hori et al9) reported that 100 mU/mL insulin attenuated the angiotensin II–induced [Ca2+]i transient in cultured rat VSMCs even in the absence of external Ca2+, and inhibited inositol triphosphate–sensitive Ca2+ mobilization in these cells. The present studies in dogs do not confirm the findings in rats of Zemel and coworkers and Tuck and coworkers that insulin stimulates sarcolemmal Ca2+ efflux and inhibits Ca2+ release from intracellular stores, respectively. These discrepancies might be explained on the basis of different species, vessel-contractile hormones, or experimental techniques used in the present studies. It is not likely that our use of a physiological (40 μU/mL) versus a supraphysiological concentration of insulin could explain these discrepancies, since 100 mU/mL insulin also failed to affect basal or 5-HT–stimulated 4Ca2+ efflux from confluent primary cultured canine femoral artery VSMCs (unpublished observations).

It has not been established that the cells used in the present study are an appropriate model to study the effects of insulin on Ca2+ transport in individual nonproliferated canine femoral artery VSMCs, since proliferation of these cells can change cell phenotype.16 Nevertheless, the finding that the effects of 5-HT and insulin on the [Ca2+]i transient were identical in both types of cells suggests that there may be important similarities in this regard. Furthermore, the confluent cultured cells have many features in common with noncultured VSMCs. For instance, they have similar acid-base transport systems, Na+-K+ pumps, and actin-to-myosin ratios as intact VSMCs.15,16,25-32

It is noteworthy that our previous studies have shown that preincubation with insulin inhibits 5-HT–induced contraction of individual nonproliferated canine femoral artery VSMCs when 5-HT is administered in the absence but not presence of verapamil. We have also showed that insulin impairs external K+–induced contraction in these cells.6 It would seem that a physiological insulin concentration inhibits contraction of dog femoral artery VSMCs, at least in part, by inhibiting voltage-operated Ca2+ channel activity. Whether this accounts for the inhibition by insulin of 5-HT–stimulated Ca2+ influx in the cells used in the present study has not yet been determined, nor has it been determined whether insulin alters the binding of 5-HT to these cells.

Acknowledgments

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