Tyrosine phosphorylation modulates arteriolar tone but is not fundamental to myogenic response

BRIAN E. SPURRELL, TIMOTHY V. MURPHY, AND MICHAEL A. HILL
Microvascular Biology Group, Department of Human Biology and
Movement Science, RMIT University, Bundoora, Victoria 3083, Australia

Spurrell, Brian E., Timothy V. Murphy, and Michael A. Hill. Tyrosine phosphorylation modulates arteriolar tone but is not fundamental to myogenic response. Am. J. Physiol. Heart Circ. Physiol. 278: H373–H382, 2000.—The present study investigated the role of protein tyrosine phosphorylation in myogenic responsiveness of rat skeletal muscle arterioles. Arterial segments were cannulated and pressurized without intraluminal flow. All vessels studied developed spontaneous tone and demonstrated significant myogenic constriction to step changes in pressure with a resultant increase in myogenic tone over an intraluminal pressure range of 50–150 mmHg. Step increases in intraluminal pressure from 50 to 120 mmHg caused a rapid and sustained elevation in intracellular \([Ca^{2+}]_i\), as measured using fura 2. Vessels with myogenic tone dilated in response to tyrosine kinase inhibitors genistein (10 or 30 \(\mu M\)) and tyrphostin A47 (10 or 30 \(\mu M\)) and constricted to the tyrosine phosphatase inhibitor pervanadate (1 or 10 \(\mu M\)). Despite the dilator effect, myogenic reactivity was not blocked by the inhibitors. Daidzein (10 \(\mu M\)), a compound structurally similar to genistein but without tyrosine kinase-inhibiting activity, did not alter vessel tone or myogenic responses. Preincubation of arterioles with genistein or tyrphostin A47 did not significantly alter baseline arteriolar \([Ca^{2+}]_i\), and neither drug reduced the increase in \([Ca^{2+}]_i\) following an acute increase in intraluminal pressure. Constriction induced by pervanadate (10 \(\mu M\)) was not accompanied by a significant increase in intracellular \([Ca^{2+}]_i\), even though removal of extracellular \(Ca^{2+}\) reversed the constriction. Examination of smooth muscle tyrosine phosphorylation, using a fluorescent phosphotyrosine antibody and confocal microscopy, showed that increased intraluminal pressure resulted in an increase in anti-phosphotyrosine fluorescence. Because manipulation of tyrosine kinase activity was found to alter vessel diameter, these data support a role for tyrosine phosphorylation in modulation of arteriolar tone. However, the results indicate that acute arteriolar myogenic constriction does not require tyrosine phosphorylation.

arteriole; cell signaling; vascular smooth muscle; microcirculation

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other mechanisms controlling the contractile state of smooth muscle cells. For example, mitogen-activated protein (MAP) kinase, a tyrosine kinase, which itself is partly activated by tyrosine phosphorylation, has been suggested to modulate the activity of the actin, and myosin-binding protein caldesmon (1). Interestingly, in conduit arteries this tyrosine kinase pathway has been shown to be activated by an applied load or stretch (10). Thus it is possible that tyrosine kinase could also be linked to the myogenic response.

On the basis of the above, the present studies were designed to examine the possible involvement of tyrosine phosphorylation mechanisms in arteriolar myogenic responsiveness. A further aim of this study was to examine the effect of manipulating tyrosine kinase and phosphatase activity on vascular smooth muscle intracellular Ca$^{2+}$.

**MATERIALS AND METHODS**

**Animals**

These studies used male Sprague-Dawley rats, age 6–9 wk and weight 200–350 g. Before experiments, rats were housed in pairs in a dedicated animal facility with a 12:12 h light-dark cycle. During this period, rats were allowed free access to a standard rat chow and drinking water. All procedures were approved by the Animal Care and Use Committee at RMIT University.

**Isolated Arteriole Preparation**

Rats were anesthetized with pentothal sodium (100 mg/kg ip) after which the right cremaster muscle was exteriorized, excised from the animal, and placed in a cooled (4°C) chamber (pRinger) after which the right cremaster muscle was exteriorized, excised from the animal, and placed in a cooled (4°C) chamber. It is possible that tyrosine kinase could also be linked to the myogenic response.

**In Situ Demonstration of Smooth Muscle Tyrosine Phosphorylation**

After inhibition of tyrosine phosphatase activity with pervanadate (100 µM), arteriole segments were fixed under pressure in a mixture of ice-cold 50% (vol/vol) ethanol-4% polyethylene glycol after pressure stimulation (see Experimental Protocols) and stored at 4°C. For antibody labelling, vessel segments were washed with phosphate-buffered saline (PBS: 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl, 8 mM Na$_2$HPO$_4$, and 2% paraformaldehyde (2g/100 ml)) and incubated with 0.1% Triton X-100 in PBS for 10 min at room temperature. Vessels were washed with PBS and then incubated in a solution containing 0.9% sodium citrate, 2% filtered goat serum, 0.05% Triton X-100, 0.025% Na$_3$ and FITC-labelled phosphotyrosine monoclonal antibody (1:50) for 45 min at 21°C. After incubation, the vessel segments were washed with 0.02% Triton X-100/0.9% sodium citrate in PBS, mounted on a glass slide in Fluomount, and examined by laser confocal microscopy (MRC-500, Bio-Rad). Quantification of the pixel intensity in confocal images of vascular smooth muscle cells was performed using the histogram-area functions of the MRC/Comos software. The regions examined were outlined manually, and the calculated mean fluorescence intensity was obtained for the selected region. Data are presented as the mean fluorescence intensity of the selected regions. In some experiments, specificity of labelling was confirmed by preabsorbing the antibody with 1 mM phosphotyrosine before incubation with the vessels. This procedure abolished >90% of the subsequent fluorescence (not shown).

**Experimental Protocols**

Effect of inhibitors of tyrosine kinase and tyrosine phosphatase on arteriolar myogenic reactivity. In each arteriole, following equilibration at 70 mmHg, intraluminal pressure was reduced to 50 mmHg for 15 min, and two consecutive pressure-diameter curves were constructed by sequential step increases in intraluminal pressure to 70, 100, 120, and 150 mmHg. Vessels were maintained at each pressure for 5 min, and the minimum intraluminal diameters were recorded. For the second pressure-diameter curve, vessels were superfused with PBS alone (control) or PBS containing the...
tyrosine kinase inhibitors genistein (10 or 30 µM) or tyrphostin A47 (10 or 30 µM), daidzein (10 µM), a compound structurally similar to genistein but without inhibitory effect on tyrosine kinase, or the tyrosine phosphatase inhibitor sodium pervanadate (1 or 10 µM). Vessels were exposed to drugs for 15 min before the second pressure-diameter curve. At the conclusion of each experiment, vessels were preincubated in Ca²⁺-free PBS containing 2 mM EGTA for 15 min, and a passive pressure-diameter curve was recorded. Diameters for the pressure-response curves have been expressed as a ratio of the diameter under a given experimental condition to that at 70 mmHg in Ca²⁺-free PBS.

As a control for the effect of vasodilation, per se on myogenic reactivity, an additional seven experiments were conducted where vessels were treated with adenosine. The concentration of adenosine was adjusted to give a vasodilation similar to that caused by the tyrosine kinase inhibitors. Adenosine was chosen because its vasoactive effects are primarily mediated through cAMP-dependent mechanisms.

Effect of inhibitors of tyrosine kinase and tyrosine phosphatase on Ca²⁺ signaling during acute arteriolar myogenic responses. After incubation with fura 2 as described previously, vessels were equilibrated at an intraluminal pressure of 50 mmHg for 15 min followed by a step increase in pressure to 120 mmHg. Vessel diameter and intracellular Ca²⁺ responses were recorded for 1 min before the pressure step and for an additional 5 min after the pressure step. In some experiments, vessels were exposed to either genistein (10 or 30 µM) or tyrphostin A47 (10 or 30 µM), and both internal vessel diameter and intracellular [Ca²⁺] were recorded for 15 min at 70 mmHg. At the conclusion of this preincubation period, intraluminal pressure was lowered to 50 mmHg for 5 min, and the response to the 50- to 120-mmHg pressure step was repeated. In experiments examining the effects of pervanadate on intracellular [Ca²⁺], vessels were subjected to a single pressure step from 50 to 120 mmHg in order to establish a myogenic response. Pressure was then reduced to 70 mmHg where it remained throughout the experiment. Before treatment with pervanadate (0.1, 1, or 10 µM), an increase in intracellular [Ca²⁺] was elicited using phenylephrine (1 µM).

In situ demonstration of tyrosine phosphorylation during pressure stimulation. Vessels (n = 24) were allowed to develop spontaneous myogenic tone while maintained at 70 mmHg and then treated with 10 µM acetylcholine. The diameter achieved in the presence of acetylcholine was recorded as an estimate of maximal diameter. The vessel was then washed and spontaneous tone was allowed to redevelop after which the intraluminal pressure was lowered to 30 mmHg for 20 min. In five of these experiments, vessels were incubated with tyrphostin A47 (30 µM). After this equilibration period, intraluminal pressure was stepped to 10, 70, or 100 mmHg. Vessels were maintained at a particular test pressure for 15 min. Pervanadate (100 µM) was added to the PBS for the final 5 min of this period to inhibit endogenous tyrosine phosphatase activity, as described previously (21). At the conclusion of the test period, vessels were fixed in the vessel chamber as described above.

Drugs and Chemicals

Genistein and daidzein (Sigma, St. Louis, MO) were prepared in DMSO as a 100 mM stock solution and frozen at −20°C for later use. Tyrphostin A47 (RG-50864, Sigma) was dissolved as 5 mg in 1 ml DMSO (22.7 mM) stock, and this solution was frozen at −20°C for later use. Control studies performed with DMSO alone showed that there were no effects of this vehicle at concentrations used in any of the protocols. Sodium pervanadate was prepared as a 50 mM stock solution using the following procedure. Two milliliters of a 100 mM sodium orthovanadate (ICN, Aurora, OH) solution were activated by mixing with 2 ml of a 100 mM hydrogen peroxide solution (30% wt/wt; Ajax Chemicals, Auburn, NSW, Australia), and the reaction was to proceed for 15 min at room temperature. The reaction was terminated by the addition of 400 U/ml catalase, and the pervanadate solution was then ready for use after an additional 15-min incubation period. Each of these drugs was further diluted in PSS. Monoclonal FITC-conjugated antiphosphotyrosine antibody was obtained from Sigma.

Statistical Analysis

Data are expressed as means ± SE. Simple comparisons of the means and SE of data were performed using Student’s t-test; multiple comparisons were determined using analysis of variance with the paired least-squares difference post hoc test. Values of P < 0.05 were considered to be significant.

RESULTS

Effect of Tyrosine Kinase and Phosphatase Inhibitors on Myogenic Tone and Contraction

The pressure-diameter curves for isolated cremaster arterioles from the rat are shown in Fig. 1. The intraluminal pressure of the vessels was increased in 20-mmHg steps over the range of 50–150 mmHg, with vessels being maintained at each pressure for at least 5 min before the steady-state diameter was recorded. As the intraluminal pressure of rat cremaster arterioles was raised, the internal diameter of the arterioles decreased as they responded to the pressure increase with a myogenic constriction and increased myogenic tone (as shown by the control pressure-diameter curve in Fig. 1A). In a nominally Ca²⁺-free PSS (0 mM Ca²⁺-2 mM EGTA), the vessels diluted passively in response to increasing intraluminal pressure (Fig. 1A). The tyrosine kinase inhibitors tyrphostin A47 (10 and 30 µM) and genistein (30 µM) significantly reduced the arteriolar smooth muscle tone, with the effect of tyrphostin A47 appearing concentration dependent. At 70 mmHg, arteriolar diameter was 47.3 ± 2.1% of the passive diameter; in the presence of tyrphostin A47 (30 µM) diameter was 70.3 ± 4.5% (P < 0.05, unpaired Student’s t-test); in the presence of genistein (30 µM) diameter was 74.5 ± 6.0% (P < 0.05, unpaired Student’s t-test; see Fig. 1B). A lower concentration of genistein (10 µM) also caused a small but significant dilation of vessels at 70 mmHg (50.9 ± 1.4%, not shown). Despite this vasodilator effect, the tyrosine kinase inhibitors did not alter the steady-state myogenic responsiveness of the arterioles (Fig. 1B). In the presence of the tyrosine kinase inhibitors, the slope of the pressure-diameter curves was not significantly different from the relationship in the absence of the drugs (Table 1). Daidzein (30 µM), a structural isomer of genistein but a significantly less potent inhibitor of tyrosine kinase, caused a significant but small decrease in vessel diameter, but in common with tyrphostin A47 and genistein did not alter the slope of the pressure-diameter curve (Table 1). The tyrosine phosphatase inhibitor pervanadate (1 and
10 µM) caused a concentration-dependent constriction of the vessels. At 70 mmHg, vessel diameter in the presence of 1 µM pervanadate was 17.0 ± 2.1% of the passive diameter (control 47.3 ± 2.1%); for 10 µM pervanadate diameter was 11.4 ± 1.0% (Fig. 1C). In the presence of pervanadate, the slope of the arteriolar pressure-diameter curve was significantly reduced (Fig. 1C; Table 1), presumably because the vessel was constricted to a high degree by pervanadate. However, vessels could still respond to an increase in intraluminal pressure. In the presence of 10 µM pervanadate, vessel diameter was reduced from 13.0 ± 0.9% of passive at 50 mmHg to 5.4 ± 0.2% of passive at 150 mmHg (Fig. 1C).

The effects of the tyrosine kinase inhibitors were compared with those of the vasodilator adenosine. The concentrations of adenosine used (1–10 µM) were adjusted, for each experiment, to evoke a similar degree of arteriolar dilation to that caused by 30 µM tyrophostin A47. Adenosine caused a mean dilation of the vessels to 68.1 ± 6.3% of passive diameter (control 46.0 ± 3.6%) at 70 mmHg (Fig. 1D). Despite being dilated in the presence of adenosine, the steady-state myogenic responsiveness of the vessels was not impaired as shown by the slope of the pressure-diameter curve being not significantly different from control values (Table 1).

The effects of the tyrosine kinase and phosphatase inhibitors on active wall tension are shown in Fig. 2. Whereas the tyrosine kinase inhibitors reduced the amount of active tension developed at each pressure (Fig. 2A), the ability of the vessels to actively increase wall tension was not altered compared with control vessels across the pressure range. This is shown by the slope of the active tension-pressure curve, being not significantly altered by tyrophostin A47 or genistein (Table 1). Similarly, pervanadate increased the amount of active wall tension at each pressure tested without altering the relationship between pressure and tension (Fig. 2B, Table 1).

Effect of Tyrosine Kinase Inhibitors Genistein and Tyrophostin A47 on Alterations in Intracellular [Ca²⁺] in Response to Increases in Intraluminal Pressure

Additional experiments examined the effects of the tyrosine kinase inhibitors on arteriolar [Ca²⁺]. Arteri- odes were incubated with the Ca²⁺-sensitive dye fura 2-AM (5 µM), and their intraluminal pressure was raised instantaneously, or "stepped," from 50 to 120 mmHg. The vessels were incubated with the tyrosine kinase inhibitors for 15 min before the pressure-step test, during which time the intraluminal pressure was maintained at 70 mmHg. During this period both concentrations of genistein (10 or 30 µM) or tyrophostin A47 (10 or 30 µM) significantly dilated the vessels as shown previously. However, neither drug significantly altered arteriolar [Ca²⁺] during this period, as indicated by the 340-to-380 nm fluorescence ratio (R340/380); genistein 30 µM: preincubation R340/380 1.06 ± 0.08; postincubation R340/380 0.88 ± 0.09, P > 0.05, paired t-test; tyrophostin A47 30 µM: preincubation R340/380

![Graphs showing effects of tyrosine kinase inhibitors on arteriolar pressures and tensions](http://ajpheart.physiology.org/)

### Table 1. Slope of pressure-diameter and pressure-active tension curves from Figs. 1 and 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pressure-Diameter Curve in Fig. 1, A–D</th>
<th>Pressure-Active Tension Curve in Fig. 2, A and B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−0.22 ± 0.02</td>
<td>4.69 ± 0.17</td>
</tr>
<tr>
<td>Ca²⁺ (0 µM)</td>
<td>+0.13 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>Tyrophostin A47 (10 µM)</td>
<td>−0.25 ± 0.06</td>
<td>4.58 ± 0.24</td>
</tr>
<tr>
<td>Tyrophostin A47 (30 µM)</td>
<td>−0.23 ± 0.03</td>
<td>4.12 ± 0.22</td>
</tr>
<tr>
<td>Genistein (30 µM)</td>
<td>−0.20 ± 0.05</td>
<td>3.69 ± 0.29</td>
</tr>
<tr>
<td>Daizdein (10 µM)</td>
<td>−0.19 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Pervanadate (1 µM)</td>
<td>−0.09 ± 0.01*</td>
<td>5.74 ± 0.35</td>
</tr>
<tr>
<td>Pervanadate (10 µM)</td>
<td>−0.07 ± 0.01*</td>
<td>5.48 ± 0.11</td>
</tr>
<tr>
<td>Adenosine (1–10 µM)</td>
<td>−0.25 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Slopes were calculated via linear regression to the equation y = mx + c, where y is diameter or active tension, x is pressure, and m is the slope of the fitted line. *Significant difference in slope from the control value (P < 0.05, Student’s t-test).
Genistein (10 µM) did not significantly alter these values (Fig. 3B). In the presence of genistein (10 µM), the normalized prestep $R_{340/380}$ was 1.02 ± 0.04. The peak poststep $R_{340/380}$ was 1.19 ± 0.05; the steady-state $R_{340/380}$ was 1.11 ± 0.04. A higher concentration of genistein (30 µM) caused a large dilation of fura 2-loaded vessels, such that they behaved passively when subjected to the 50- to 120-mmHg pressure step. This appeared to be a nonspecific effect related to treating vessels with both fura 2 and genistein. Despite the lack of an observable myogenic response under these conditions, the pressure step still evoked an increase in arteriolar $[\text{Ca}^{2+}]$ not significantly different from the control response (prestep normalized $R_{340/380}$ 0.98 ± 0.05; peak 1.18 ± 0.10; steady state 1.14 ± 0.09).

Tyrphostin A47 (10 µM) significantly diluted the vessels and attenuated the myogenic contraction associated with the 50- to 120-mmHg pressure step (Fig. 4). Like genistein, tyrphostin A47 did not inhibit the increase in arteriolar $[\text{Ca}^{2+}]$ associated with the pressure step (Fig. 4B). In these experiments control normalized prestep $R_{340/380}$ was 1.00 ± 0.01; after the pressure

$1.09 ± 0.06$; postincubation $R_{340/380}, 1.09 ± 0.05, P > 0.05, \text{paired t-test}$. The step increase in pressure from 50 to 120 mmHg caused a transient, pressure-induced distention followed by vasoconstriction to a diameter significantly smaller than at 50 mmHg (Figs. 3A and 4A).

The pressure-induced distention-constriction was accompanied by a biphasic increase in arteriolar $[\text{Ca}^{2+}]$, as indicated by the $R_{340/380}$ (Figs. 3B and 4B). A rapid, transient increase in $[\text{Ca}^{2+}]$ was observed, which peaked ~60 s after the increase in pressure. For the 4 min following the peak increase, $[\text{Ca}^{2+}]$ remained elevated above the prestep level but declined to a steady-state level (Figs. 3B and 4B). In the absence of extracellular $\text{Ca}^{2+}$, the resting intracellular $[\text{Ca}^{2+}]$ of vessels maintained at 50 mmHg was significantly reduced and stepping the intraluminal pressure to 120 mmHg failed to induce a myogenic contraction or raise intracellular $[\text{Ca}^{2+}]$ (Figs. 3 and 4).

Genistein (10 µM) diluted the vessels but did not attenuate the contractile response associated with the pressure-step or the subsequent increase in $[\text{Ca}^{2+}]$ (Fig. 3). In $[\text{Ca}^{2+}]$ measurements the control prestep $R_{340/380}$ normalized to the $R_{340/380}$ during the 60-s period before the pressure step was 0.99 ± 0.01; the peak $R_{340/380}$, which occurred ~60 s after the pressure step, was 1.21 ± 0.05 ($P < 0.05$; Fig. 3B). The steady-state level $R_{340/380}$ was 1.13 ± 0.03, 5 min after the step (Fig. 3B).
step to 120 mmHg, peak $R_{340/380}$ was 1.25 ± 0.03 and steady-state $R_{340/380}$ was 1.15 ± 0.02. In the presence of tyrphostin A47 (10 µM), these values were 1.02 ± 0.03, 1.31 ± 0.07, and 1.26 ± 0.08, respectively. Also as with genistein, a higher concentration of tyrphostin A47 (30 µM), in the presence of fura 2 almost fully dilated the vessels; under these conditions the vessels behaved passively in response to the pressure step, and no myogenic contraction was observed. But an increase in arteriolar $[Ca^{2+}]$ in response to the pressure step was still observed. In the presence of tyrphostin A47 (30 µM) prestep $R_{340/380}$ was 1.07 ± 0.05 and peak was 1.35 ± 0.08, values not significantly different from control. However, steady-state $R_{340/380}$ was 1.35 ± 0.10, a value significantly greater than control ($P < 0.05$, Student's t-test; Fig. 4B).

Effect of Tyrosine Phosphatase Inhibitor Pervanadate on Alterations in Arteriolar Diameter and Intracellular $[Ca^{2+}]$ in Response to Increases in Intraluminal Pressure

In vessels maintained at 70 mmHg, pervanadate (0.1, 1, and 10 µM) caused a concentration-dependent reduction in diameter (Fig. 5A). This constriction was not accompanied by a significant increase in intracellular $[Ca^{2+}]$, as measured by $R_{340/380}$ (Fig. 5B). Pre-pervanadate diameter was 58.1 ± 4.5% of passive, and post-pervanadate (10 µM) diameter was 24.5 ± 5.5%; pre-pervanadate $R_{340/380}$ was 0.88 ± 0.05; post-pervanadate (10 µM), $R_{340/380}$ was 0.83 ± 0.03, $n=5$ for all ($P >$
0.05, paired t-test; Fig. 5B). In the same series of experiments a rapid, transient increase in intracellular [Ca$^{2+}$] accompanied the constrictions caused by both phenylephrine (1 µM) and a pressure step from 50 to 120 mmHg (latter not shown). Post-phenylephrine diameter was 32.5 ± 4.8% of passive; post-phenylephrine R$_{300/380}$ was 1.76 ± 0.38 (P < 0.05, paired t-test; n = 4 for all, Fig. 5, A and B). Removal of extracellular Ca$^{2+}$ from the PSS completely reversed the pervanadate (10 µM)-induced constriction (diameter 96.6 ± 1.2% of passive; n = 5; Fig. 5C). A higher concentration of pervanadate (100 µM) failed to constrict arterioles bathed in Ca$^{2+}$-free PSS (not shown).

Effect of Increases in Intraluminal Pressure on Tyrosine Phosphorylation in Arterioles Measured Using Confocal Microscopy

In these experiments vessels were stepped from 30 mmHg to 10, 70, or 100 mmHg, as described in the protocols and procedures. After being stepped to 10, 70, or 100 mmHg, vessels were maintained at the test pressure for 15 min and incubated with pervanadate (100 µM) for the final 5 min of this period. Vessels were then fixed, incubated with a fluorescently labelled antibody to phosphotyrosine, and examined using a laser confocal microscope. Fluorescence intensity significantly increased with increasing intraluminal pressure (Fig. 6C). The mean diameter of vessels maintained at 30 mmHg was 68.4 ± 1.1% of passive (n = 19). Vessels stepped from 30 to 70 or 100 mmHg responded with a myogenic contraction and maintained myogenic tone (Fig. 6B). Reducing intraluminal pressure from 30 to 10 mmHg also reduced the vessel diameter; however, this was a result of passive collapse rather than active contraction (Fig. 6B). In vessels incubated with tyrphostin A47 (30 µM) and subsequently stepped to 100 mmHg, the increase in fluorescence intensity was significantly reduced compared with control tissues (P < 0.05, Student’s t-test; Fig. 6A).

**DISCUSSION**

In the presence of the tyrosine kinase inhibitors genistein or tyrphostin A47, rat cremaster arterioles retained the ability to constrict in response to alterations in intraluminal pressure and, furthermore, the increase in smooth muscle intracellular [Ca$^{2+}$], which follows an acute increase in intraluminal pressure, was not reduced by genistein or tyrphostin A47. However, the tyrosine kinase inhibitors and the tyrosine phosphatase inhibitor pervanadate were able to dilate and constrict the vessels, respectively. These observations suggest that increased protein tyrosine kinase activity is not fundamental to acute myogenic constriction but may play a role in modulating smooth muscle tone of pressurized arterioles.

Genistein and tyrphostin A47 are structurally unrelated tyrosine kinase inhibitors with pharmacologically distinct mechanisms of action. Genistein exerts its action by inhibiting the ATP-binding site, whereas tyrphostin interacts with the substrate binding site of the enzymes. Studies in rat aorta and porcine coronary artery showed that 30 µM genistein fully inhibited the contractile action of tyrosine kinase-activating agents such as angiotensin II or the tyrosine phosphatase inhibitor pervanadate and their ability to generate phosphotyrosine residues on proteins in these tissues (14, 15). In the present study both genistein and tyrphostin A47 caused a concentration-dependent dilatation of vessels exhibiting myogenic tone, whereas daidzein, an inactive analog of genistein, had a small constricting effect inconsistent with inhibition of tyrosine kinase. Concentrations of inhibitors not causing maximal dilation failed to alter the ability of vessels to respond with an acute myogenic constriction as intraluminal pressure was raised. In contrast to the dilator effects of the tyrosine kinase inhibitors, the tyrosine phosphatase inhibitor pervanadate (14) caused a marked concentration-dependent constriction of arterioles. Under these conditions the myogenic pressure-diameter curve was reduced, probably because of the extent of constriction induced by the inhibitor. Further evidence of the inability of the drugs to alter the acute myogenic responsiveness of the vessels is shown by examination of the relationship between vessel trans-
mural pressure and active wall tension. The tyrosine kinase inhibitors caused a “downward shift” of this relationship, dilating the vessel without altering the proportionate increase in active tension developed in response to each pressure step. Similarly, pervanadate caused an upward shift of the active tension-pressure curve, suggesting that the dilator and constrictor activities of these compounds did not alter the mechanisms fundamental to myogenic responses.

Consistent with previous studies (35), myogenic responses were abolished by removal of extracellular Ca\(^{2+}\), and in the presence of Ca\(^{2+}\) an acute increase in intraluminal pressure caused a rapid and sustained increase in (relative to basal) in vascular smooth muscle intracellular [Ca\(^{2+}\)]. Ca\(^{2+}\) entry during myogenic constriction is thought to occur largely through activation of cell membrane voltage-sensitive Ca\(^{2+}\) channels (18, 30). Several studies have shown that the dilator effects of tyrosine kinase inhibitors on smooth muscle are accompanied by a reduction in intracellular [Ca\(^{2+}\)], most probably caused by inhibition of Ca\(^{2+}\) influx. In rat mesenteric arteries (28) genistein inhibited the norepinephrine-induced increases in [Ca\(^{2+}\)], and in smooth muscle cells from porcine coronary artery, genistein inhibited [Ca\(^{2+}\)] increases caused by endothelin (15). In the dog mesenteric artery and saphenous vein (17), genistein and tyrphostin A47 selectively inhibited the component of phenylephrine-induced contractions reliant on extracellular Ca\(^{2+}\). In vascular smooth muscle cells from the rabbit ear artery, tyrphostins inhibited voltage-sensitive Ca\(^{2+}\) channels (31). From these findings, it seemed possible that tyrosine kinase inhibitors dilated the vessels from the rat cremaster by inhibiting Ca\(^{2+}\) influx caused by increased transmural pressure.

In the present studies, neither genistein nor tyrphostin A47 altered the increase in arteriolar intracellular [Ca\(^{2+}\)] caused by an acute 50- to 120-mmHg increase in intraluminal pressure. Tyrophostin A47 reduced the myogenic constriction associated with the pressure step; however, this was possibly due to an additive effect of a combination of fura 2 loading together with the kinase inhibitor, because diameter responses were not affected by the inhibitors in the absence of fura 2 (Fig. 1). During the incubation phase before the pressure-step test in which the vessels were maintained at 70 mmHg, neither genistein nor tyrphostin A47 reduced intracellular [Ca\(^{2+}\)] despite causing a significant dilation of the arterioles. These results suggest that the vasodilator effects of the tyrosine kinase inhibitors on cremaster arterioles are not associated with a reduction in intracellular [Ca\(^{2+}\)], and tyrosine kinase inhibition does not prevent the pressure-induced influx of Ca\(^{2+}\) through voltage-sensitive Ca\(^{2+}\) channels and the subsequent contraction. This is in contrast to other studies (8, 15, 24, 34) mentioned earlier, where tyrosine kinase inhibition reduced both the constriction and increase in intracellular [Ca\(^{2+}\)] caused by agents such as norepinephrine, phenylephrine, endothelin, and angiotensin II. Thus it would appear that cellular stretch and the increase in wall tension, which initiates the myogenic response, may activate Ca\(^{2+}\) entry through a tyrosine kinase-independent mechanism, distinct from the receptor-mediated actions of vasoactive agents.

Further studies examined the Ca\(^{2+}\) dependency of pervanadate-induced contractions. Pervanadate did not increase intracellular [Ca\(^{2+}\)] despite causing significant arteriolar constriction. Such contractions were of similar magnitude as those caused by raising the intraluminal pressure or by the \(\alpha\)-adrenoceptor agonist phenylephrine, both of which were associated with significant increases in intracellular [Ca\(^{2+}\)]. Together with the (lack of) effect of tyrosine kinase inhibitors on arteriolar [Ca\(^{2+}\)], these results suggest that altering the level of tyrosine phosphorylation within the cremaster arteriole brings about a change in the level of muscular tone chiefly by altering mechanism(s) other than the level of intracellular [Ca\(^{2+}\)] per se. It should be noted that the contraction caused by pervanadate still required Ca\(^{2+}\) because it was abolished by the removal of extracellular Ca\(^{2+}\). These findings are consistent with those obtained with the tyrosine kinase inhibitors, suggesting that interaction with Ca\(^{2+}\) entry does not play a part in the effect of tyrosine phosphorylation on cremaster arteriole tone. Complementing studies with the tyrosine kinase inhibitors, there is strong evidence from previous studies that tyrosine phosphatase inhibitors, including pervanadate, increase the activity of voltage-sensitive Ca\(^{2+}\) channels in vascular smooth muscle cells. Pervanadate-induced contractions of rat aorta were abolished by removal of extracellular [Ca\(^{2+}\)] or the voltage-sensitive Ca\(^{2+}\)-channel antagonist nifedipine (14), and tyrosine phosphatase inhibitors, including pervanadate, activated voltage-sensitive Ca\(^{2+}\)-channels in isolated smooth muscle cells (32).

Given the lack of effect of the tyrosine kinase inhibitors on intracellular [Ca\(^{2+}\)] and the myogenic response, it is conceivable that the drugs were able to dilate and constrict the arteriole by affecting another part of the contractile pathway. The mechanism through which vascular smooth muscle cells respond to an increase in vessel wall tension and/or cell membrane stretch is not fully understood. A series of recent studies (4, 20) demonstrated that inhibition of integrin binding with RGD peptides results in vasodilation and decreased levels of intracellular Ca\(^{2+}\). Furthermore, a variety of cell studies have shown that focal adhesions, through which cells communicate with the extracellular matrix via integrin binding, represent a site rich in tyrosine kinase activity (3, 23, 26). Increased tyrosine phosphorylation specifically related to mechanotransduction has been shown in stretched cardiac myocytes (25) and endothelial cells subjected to shear stress (21). In addition, contractile pathways that may function in parallel to the classic Ca\(^{2+}\)-calmodulin-myosin light chain kinase mechanism, such as that utilizing MAP kinase, are activated by tyrosine phosphorylation of the constituent proteins (1, 10). It is possible that genistein, tyrphostin, and pervanadate interact with Ca\(^{2+}\)-independent components of the contractile pathway, which are not involved in the myogenic response, or mechanisms having a lower Ca\(^{2+}\) requirement than the
classical myosin light chain phosphorylation pathway, which involve tyrosine phosphorylation (8).

Tyrosine phosphorylation was examined more directly by fixing vessels maintained at different intraluminal pressures and subsequently incubating them with a fluorescently labelled antibody to phosphotyrosine. This technique has been used previously to demonstrate changes in tyrosine phosphorylation in endothelial cells of intact arterioles (21). In the present study, there was an increase in tyrosine phosphorylation in the smooth muscle cells of the vessel wall associated with increasing pressure and myogenic tone. Because tyrosine phosphatase was inhibited in these experiments by a high concentration of pervanadate, the increase in tyrosine phosphorylation with intraluminal pressure is best explained by a corresponding increase in protein tyrosine kinase activity rather than a decrease in tyrosine phosphatase activity. Prior application of the tyrosine kinase inhibitor tyrphostin A47 inhibited the increase in anti-phosphotyrosine fluorescence caused by elevated pressure and pervanadate, supporting the idea that tyrosine kinase activity is responsible for the rise in phosphotyrosine. The observations that pervanadate constricted the vessels to the same degree regardless of the intraluminal pressure and the level of phosphorylation increased with intraluminal pressure, further suggest that the data are not an artifact of the collection-fixation procedure.

Methodological considerations relating to the use of the phosphotyrosine antibody are that this approach does not provide any information regarding actual proteins undergoing phosphorylation nor does it provide any direct indication of the functional significance of this modification. Because tyrosine phosphorylation is utilized in a variety of signalling pathways in addition to those mediating contraction, it is conceivable that the increase in fluorescence relates to the activation of an unrelated pathway. For example, the increase in fluorescence, or a component thereof, may be involved in a mechanism underlying pressure-induced growth or modification of the arterial wall. The data do, however, indicate that the level of tyrosine kinase activity is modulated by the level of intraluminal pressure or a related variable.

In summary, these studies demonstrate that tyrosine kinase activity is elevated in rat cremaster arterioles with increasing myogenic tone. Tyrosine kinase activity is not required for myogenic responses because inhibition of tyrosine kinase did not prevent myogenic contractions in response to an acute increase in intraluminal vessel pressure or the increase in intracellular [Ca^{2+}] associated with that response. However, inhibition of tyrosine kinase diluted myogenically active vessels, with this effect occurring independently of alterations in vascular smooth muscle intracellular [Ca^{2+}]. Constriction of arteries caused by the tyrosine phosphatase inhibitor pervanadate were not associated with an increase in intracellular [Ca^{2+}], and therefore other mechanisms may be of greater importance in the effect on vascular tone of altered tyrosine phosphorylation. The tyrosine kinase and phosphatase inhibitors may be affecting a parallel pathway involved in the maintenance of myogenic tone aside from Ca^{2+} entry and myosin light chain phosphorylation, such as the MAP kinase pathway or the activation of focal adhesions caused by cell stretching. The identity of the proteins phosphorylated by protein tyrosine kinases in this preparation and the interaction between tyrosine phosphorylation and intracellular [Ca^{2+}] await further investigation.

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Address for reprint requests and other correspondence: M. A. Hill, Dept. of Human Biology and Movement Science, RMIT Univ., Plenty Rd., Bundoora, Victoria 3083, Australia (E-mail: MA.Hill@RMIT.EDU.AU).

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