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Mechanisms underlying pervanadate-induced contraction of rat cremaster muscle arterioles

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Abstract

The current study examined the role of extracellular Ca^{2+}, calmodulin and myosin light-chain kinase (MLCK) in pervanadate-induced constriction of cannulated, pressurized rat cremaster arterioles. Pervanadate (0.03–100 μM) induced a concentration-dependent constriction of arterioles that was significantly attenuated ($P<0.05$) by the tyrosine kinase inhibitor tyrphostin 47 (30 μM). The L-type voltage-sensitive Ca^{2+} channel antagonists verapamil (10 μM) and nifedipine (1 μM) dilated vessels possessing myogenic tone but had no demonstrable effect on pervanadate constriction, while a higher concentration of nifedipine (10 μM) reduced constriction by approximately 50%. Pervanadate-induced contractions were reduced by the calmodulin inhibitor W-7 (*N*-(6-aminohexyl)-chloro-1-naphtalene sulphonamide, 50 μM) and the MLCK inhibitor ML-7 (1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine, 10 or 30 μM). Removal of extracellular Ca^{2+} abolished the contractile effect of pervanadate. Measurement of changes in arteriolar wall $[Ca^{2+}]$ using the Ca^{2+} sensitive dye Fura-2 showed that pervanadate did not increase $[Ca^{2+}]$ during arteriolar constriction. These observations suggest that pervanadate-induced contraction of smooth muscle in the cremaster arteriole involves Ca^{2+}/calmodulin-dependent myosin phosphorylation and possibly sensitization of the contractile apparatus to Ca^{2+}. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Smooth muscle, Arteriolar; Pervanadate; Tyrosine phosphorylation; Ca^{2+}; Nifedipine; Verapamil; Myosin light-chain kinase

1. Introduction

Peroxidized sodium orthovanadate, or pervanadate, is a relatively selective inhibitor of tyrosine phosphatases (Heffetz et al., 1990; Posner et al., 1994; Bevan et al., 1995). Pervanadate stimulates contraction of smooth muscle through inhibition of tyrosine phosphatases (Laniyonu et al., 1994; Di Salvo et al., 1997); however relatively few studies have examined the contractile effect of pervanadate or other tyrosine phosphatase inhibitors in vascular smooth muscle, an area of interest given the high level of tyrosine kinase activity in this tissue (Di Salvo et al., 1989, 1994; Elberg et al., 1995). Further, the mechanism through which increased protein tyrosine phosphorylation stimulates contraction of vascular smooth muscle is largely unknown (Hughes and Wijetunge, 1998) and therefore investigation of pervanadate-induced contraction may provide an insight into the role of tyrosine phosphorylation in this tissue.

Pervanadate-induced contraction of vascular and other smooth muscle has been ascribed to an increase in intracellular $[Ca^{2+}]$, principally increased Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Removal of extracellular Ca^{2+} or the presence of the L-type voltage-sensitive Ca^{2+} channel (VSCC) antagonist nifedipine abolished pervanadate induced contraction of rat aorta and gastric fundus (Laniyonu et al., 1994). Studies in smooth muscle cells isolated from rabbit ear artery and rat basilar artery showed that pervanadate and other tyrosine phosphatase inhibitors increased current through VSCCs (Wijetunge et al., 1998; Kimura et al., 2000), together with increased tyrosine phosphorylation of the channel proteins. Thus, modulation of Ca^{2+} influx through VSCCs may be an important mechanism of tyrosine phosphorylation-induced smooth muscle contraction. In support of this, other studies performed in cells from rat aorta (Sandirasegarane and Gopalakrishnan, 1995) and A7r5 vascular smooth muscle cells (Kaplan and Di Salvo, 1996) have shown that orthovanadate increases intracellular $[Ca^{2+}]$. Orthovanadate has a variety of pharmacological actions that impact on intracellular $[Ca^{2+}]$ in addition to inhibiting tyro-

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sine phosphatase, for example, inhibition of Na^+/K^+ ATPase and sarcolemmal Ca^{2+} ATPase; however, in these two studies, orthovanadate's effects on Ca^{2+} were abolished by a tyrosine kinase inhibitor. Further, a number of studies in smooth muscle of the gastrointestinal tract (Di Salvo et al., 1993; Hatakeyama et al., 1996; Sim and Kim, 1998) and urinary tract (Alcon et al., 2000) have also suggested that increased Ca^{2+} entry into cells is an integral part of the contractile effect of tyrosine phosphatase inhibition.

Our recent study using arterioles from the rat cremaster muscle suggested that alterations in arteriolar wall $[Ca^{2+}]$ played little part in arteriolar constriction or dilation resulting from tyrosine phosphatase or kinase inhibition, respectively (Spurrell et al., 2000). These observations are consistent with the possibility that protein tyrosine phosphorylation effected arteriolar diameter through a mechanism apart from directly modulating smooth muscle intracellular $[Ca^{2+}]$. However, removal of Ca^{2+} from the extracellular bathing fluid abolished pervanadate-induced contractions, suggesting a basic requirement for Ca^{2+} in the effect. On that basis, the present study further investigated the Ca^{2+} dependence of pervanadate-induced contractions of the rat cremaster arteriole and in addition examined the role of calmodulin and myosin light-chain kinase (MLCK) in pervanadate contraction, these being mediators of Ca^{2+}-induced vascular smooth muscle contraction (Horowitz et al., 1996; Somlyo and Somlyo, 2000).

2. Materials and methods

2.1. Arteriole preparation

Male Sprague–Dawley rats (200–350 g) were anaesthesized with sodium thiopentone, 100 mg/kg i.p.). Both cremaster muscles were excised and pinned flat in a cooled (4 °C) chamber containing dissection buffer (all concentrations in mM: 3-*N*-morpholino propanesulfonic acid (MOPS) 3; NaCl 145; KCl 5; $CaCl_2$ 2.5; $MgSO_4$ 1; NaH_2PO_4 1; EDTA 0.02; pyruvate 2 and glucose 5 plus 1% (w/v) bovine serum albumin). From one muscle, a section of the first-order cremaster arteriole (1A) was dissected free of surrounding tissue. The arteriole was then cannulated at each end on glass micropipettes (tip diameter approx. 50 μm) filled with modified Krebs solution (all concentrations in mM: 111 NaCl; 25.7 $NaHCO_3$; 4.9 KCl; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 1.2 KH_2PO_4; 11.5 glucose and 10 HEPES) mounted in a superfusion chamber. The chamber was then placed on the stage of an inverted microscope and the vessel segments superfused with Krebs solution (34 °C, 4 ml/min). Krebs solution was gassed with 95% N_2/5% CO_2. The transmural pressure of the arteriole was gradually increased to 70 mmHg during which time the vessel developed myogenic tone before commencing the experiment. Arteriole segments that failed to develop myogenic tone were not used for subsequent experiments.

2.2. $[Ca^{2+}]$ and vessel diameter measurements

For measurement of changes in intracellular $[Ca^{2+}]$, vessels were incubated with 2.5 μM Fura-2-acetoxymethyl ester (Fura-2-AM; Molecular Probes, Eugene, OR) for 60 min at room temperature in Krebs containing 0.5% dimethyl sulphoxide (DMSO) and 0.01% Pluronic F-127. The abluminal surface of the vessel segment was exposed to the Fura-2-AM solution to restrict dye loading to the vascular smooth muscle layer. The dye loading procedure was followed by a 30-min washout period with warmed, fresh Krebs solution. Fura-2-loaded vessels were epi-illuminated (75 W xenon source) with light of alternating excitation wavelengths (340 and 380 nm) using a computer-controlled filter wheel. Fluorescent images were transferred from the microscope (Nikon Fluor × 20 objective, n.a. 0.75) to the imaging system using an image intensifier (Videoscope International, Washington, DC) and a charge-coupled device (CCD; Hamematsu, Bridgewater, NJ). Simultaneous transillumination with wavelengths >610 nm provided a non-fluorescent image, which enabled simultaneous measurement of internal arteriolar diameter and monitoring of the preparation while fluorescent images were collected. This procedure did not interfere with measurements of Ca^{2+}-related fluorescence. Fluorescent image intensities were expressed as the 340- to 380-nm ratio to allow quantitative estimates of changes in arteriolar wall intracellular Ca^{2+}. Details of these procedures have been published previously (Meininger et al., 1991).

2.3. Experimental protocols

2.3.1. Effects of tyrphostin 47, forskolin, Ca^{2+} channel blockers, W-7 and ML-7 on pervanadate responses

The role of tyrosine kinases in pervanadate-induced contraction was established using the tyrosine kinase inhibitor tyrphostin 47. Vessels maintained at 70 mmHg were subjected to two 'priming doses' of 3 μM pervanadate, as suggested by Laniyonu et al. (1994). The arteriole was then washed with Krebs. After re-establishing baseline diameter, a concentration–response curve was constructed to pervanadate (0.03–100 μM), after which the arterioles were washed with pervanadate-free Krebs solution. The arteriole was exposed to each concentration of pervanadate for a maximum of 5 min, or until the constriction had peaked. The vessel was then superfused with Krebs containing tyrphostin 47 (30 μM) alone for 20 min, at which point a second pervanadate concentration-response was constructed in the continued presence of tryphostin 47. At the conclusion of the experiment, the vessels were superfused with Krebs solution containing 0 mM $CaCl_2$ and 2 mM EGTA for 20 min, to establish the maximum passive diameter of the arteriole at 70 mmHg. In four experiments, vessels were superfused with this 'Ca^{2+} free' Krebs solution for 20 min following the first pervanadate concentration–response curve, then a second pervanadate concentration–response curve performed in the

presence of the Ca^{2+}-free solution, to investigate the Ca^{2+} dependency of the pervanadate-induced constriction.

The role of VSCCs in pervanadate-induced constriction was assessed using the VSCC blockers nifedipine (1 or 10 μM) or verapamil (10 μM). The experiments were performed as outlined in the preceding paragraph, with the VSCC antagonists replacing tyrphostin. Similar studies were performed with the calmodulin inhibitor W-7 (*N*-(6-aminohexyl)-chloro-1-naphtalene sulphonamide, 50 μM) and myosin light-chain kinase inhibitor ML-7 (1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine, 10 or 30 μM) to investigate the role of these entities in pervanadate-induced constriction. In four experiments, the effect of the adenylate cyclase activator (and vasodilator) forskolin (0.1 μM) on pervanadate responses was examined, to establish whether vasodilation alone altered the effect of pervanadate.

2.3.2. Effect of pervanadate and phenylephrine on arteriolar wall [Ca^{2+}]

Arterioles were prepared and incubated with Fura-2 for measurement of changes in Ca_i^{2+} as described above. Constrictor and Fura-2 fluorescence responses to pervanadate (0.1, 1 or 10 μM) were obtained, then the arterioles were washed and the diameter and Ca^{2+} response to phenylephrine (1 μM) was obtained. Finally, arterioles were washed with Ca^{2+}-free Krebs solution as described above.

2.4. Drugs and vehicles

Pervanadate was prepared by mixing 2 ml 0.1 M sodium orthovanadate with 2 ml 0.1 M hydrogen peroxide solution for 15 min. At the conclusion of this period, 200 μg catalase was added and the solution left to stand for a further 15 min. This gave a final pervanadate concentration of 50 mM. Tyrphostin 47, forskolin and ML-7 were initially dissolved in DMSO; nifedipine, verapamil and W-7 were dissolved in ethanol and diluted further in Krebs solution. Phenylephrine was dissolved in water and subsequently diluted in Krebs solution. Drugs were obtained from Sigma (Missouri, USA) with the exception of ML-7 and W-7 (Biomol, Pennsylvania, USA).

2.5. Statistics

Data were analysed using a two-way analysis of variance (ANOVA) and if significant differences were found, this was followed by a Dunnett's test for differences between individual groups of data. Values $P < 0.05$ were taken as indicative of a significant difference.

3. Results

3.1. Pervanadate responses

The average passive diameter of vessels used in this study (measured in modified Kreb's solution containing no added Ca^{2+} and 2 mM EGTA) was 153.5 ± 2.5 μm at 70 mmHg ($n = 34$). In Ca^{2+} containing Krebs solution, arterioles possessed spontaneous active tone with a diameter of 85.3 ± 4.6 μm, or 56 ± 3% of the passive diameter.

Pervanadate caused concentration-dependent constriction of cremaster arterioles over the range 0.03–100 μM (Figs. 1 and 2), with a pEC_{50} of 5.82 ± 0.17 ($n = 34$). The minimum diameter reached was 20.3 ± 1.6 μm, or 13 ± 1% of passive diameter. In four time-control experiments, there were no significant differences between the first and second concentration–response curves (not shown); therefore, in subsequent experiments, the first concentration–response curve was used as a control.

3.2. Effect of tyrphostin on responses to pervanadate

The tyrosine kinase inhibitor tryphostin 47 (30 μM) greatly inhibited contractions to pervanadate across the entire concentration range (Fig. 2). Tyrphostin also reduced the spontaneous myogenic tone of the arterioles, to 95.0 ± 1.2% of passive for these vessels (Fig. 2). The inhibition of pervanadate constructions by tyrphostin was not due to its dilatory effect on the vessels as forskolin (0.1 μM), which produced a dilation of similar magnitude to tyrphostin, did not alter the contractile effect of pervanadate (Fig. 2, Table 1).

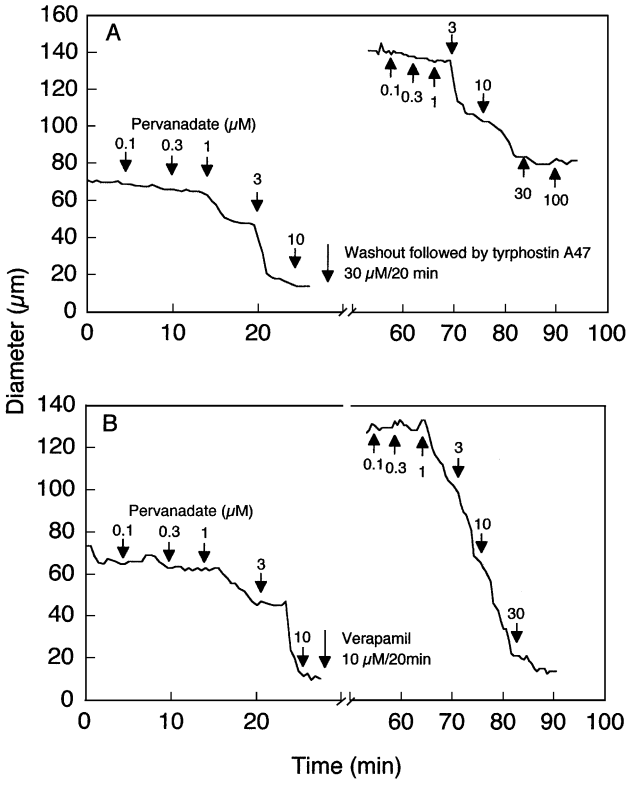

Fig. 1. Sample recording of the effect of pervanadate (0.03–100 μM) on the internal diameter of cannulated, pressurized rat cremaster arterioles. Consecutive concentration–response curves were constructed, with the second curve obtained in the presence of tyrphostin 47 (A) or the VSCC antagonist verapamil (B).

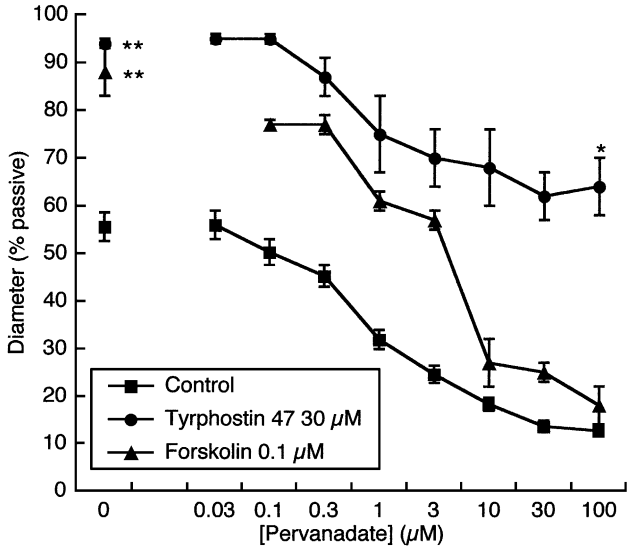

Fig. 2. Effect of tyrphostin 47 (30 μM) or forskolin (0.1 μM) on pervanadate-induced contractions of pressurized rat cremaster arterioles. Consecutive, cumulative concentration–response curves to pervanadate were constructed and tyrphostin or forskolin, where present, were added to the vessel bathing solution 20 min before the construction of the second curve. Points represent the mean ± S.E.M. * indicates a significant reduction in the maximum contraction to pervanadate ($P<0.05$, ANOVA followed by Dunnett's test). ** indicates a significant effect of tyrphostin of forskolin on the diameter of the vessels in the absence of pervanadate ($P<0.05$, Dunnett's test). ■ Control, $n=35$; ● tyrphostin 47, $n=6$; ▲ forskolin, $n=4$.

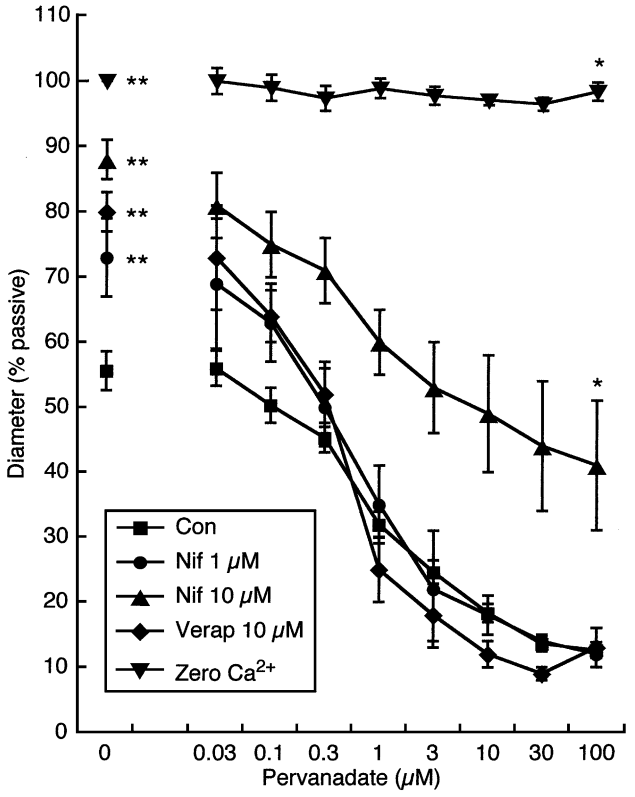

Fig. 3. Effect of the Ca^{2+} channel antagonist nifedipine 1 or 10 μM (Nif) and verapamil 10 μM (Verap) or Ca^{2+}-free Krebs solution (Zero Ca^{2+}) on pervanadate-induced contractions of pressurized rat cremaster arterioles. Consecutive, cumulative concentration–response curves to pervanadate were constructed and the drugs, where present, were added to the vessel bathing solution 20 min before the construction of the second curve. Points represent the mean ± S.E.M. * indicates a significant reduction in the maximum contraction to pervanadate ($P<0.05$, ANOVA followed by Dunnett's test). ** indicates a significant effect of nifedipine, verapamil or Ca^{2+}-free Krebs on the diameter of the vessels in the absence of pervanadate ($P<0.05$, Dunnett's test). ■ Control, $n=21$; ● nifedipine 1 μM, $n=4$; ▲ nifedipine, 10 μM, $n=8$; ♦ verapamil 10 μM, $n=4$, ▼ Zero Ca^{2+}, $n=4$.

3.3. Effect of Ca^{2+} antagonists on responses to pervanadate

The effects of the L-type Ca^{2+} channel antagonists nifedipine (1 or 10 μM) and verapamil (10 μM) on arteriolar diameter and responses to pervanadate are shown in Table 1 and Fig. 3. The lower concentration of nifedipine (1 μM) did not significantly alter the contractile response of the vessels to pervanadate, in terms of either the maximum contraction or pEC_{50} (Table 1; Fig. 3). The higher concentration of nifedipine used, 10 μM, significantly dilated the arterioles maintained at 70 mmHg (Table 1), abolishing approximately 86% of the myogenic tone. This concentration of nifedipine also attenuated the maximum pervanadate contraction to 64.1 ± 15.6 μm, or 41 ± 10% of the passive diameter (Fig. 3). However, the pEC_{50} of pervanadate was not significantly altered by 10 μM nifedipine (Table 1). Verapamil (10 μM) also dilated vessels possessing myogenic tone (Table 1); however, it did not alter the maximum contractile response to pervanadate or its pEC_{50} (Fig. 3; Table 1). Pervanadate-induced constriction was totally abolished in arterioles superfused with nominally Ca^{2+}-free Krebs solution (containing no added $CaCl_2$ and 2 mM EGTA; Fig. 3).

Table 1
Effect of tyrphostin, forskolin, voltage-sensitive Ca^{2+} channel (VSCC) antagonists, the calmodulin inhibitor W-7 and the MLCK inhibitor ML-7 on arteriolar diameter and pEC_{50} of pervanadate for contraction of rat cremaster muscle arterioles

Drug	n	Diameter (percentage passive at 70 mmHg)	pEC_{50} pervanadate
None (control)	35	57 ± 3	5.82 ± 0.17
Tyrphostin 47 (30 μM)	6	94 ± 1[a]	4.03 ± 0.72[a]
Forskolin (0.1 μM)	4	89 ± 5[a]	5.79 ± 0.44
Nifedipine (1 μM)	3	73 ± 6[a]	6.04 ± 0.14
Nifedipine (10 μM)	7	88 ± 3[a]	5.66 ± 0.36
Verapamil (10 μM)	4	80 ± 3[a]	6.42 ± 0.09
W-7 (50 μM)	5	83 ± 8[a]	4.91 ± 0.43[a]
ML-7 (10 μM)	5	77 ± 7[a]	5.56 ± 0.42
ML-7 (30 μM)	5	96 ± 3[a]	3.72 ± 0.85[a]

Diameter is expressed as a percentage of the passive (maximum) diameter determined in the same arteriole in Ca^{2+}-free Krebs solution containing 2 mM EGTA.

[a] Indicates a significant dilation of the arterioles or a significant increase in the pEC_{50} of pervanadate caused by the VSCC antagonist ($P<0.05$, ANOVA followed by Dunnett's test).

3.4. Alterations in arteriolar [Ca^{2+}] caused by pervanadate and phenylephrine

Constriction of rat cremaster arterioles caused by pervanadate (10 μM) was not accompanied by a significant increase in arteriolar wall [Ca^{2+}] (Fig. 4). In contrast, constrictions caused by the α_1-adrenoceptor agonist phenylephrine (1 μM) were associated with a significant increase in [Ca^{2+}], approximately double the pre-phenylephrine level (Fig. 4). The magnitude of contraction caused by phenylephrine was similar to that stimulated by 10 μM pervanadate. Pervanadate (100 μM) did not contract the vessels in the absence of extracellular Ca^{2+}, under which circumstances arteriolar [Ca^{2+}] was significantly reduced (Fig. 4).

3.5. Effect of W-7 and ML-7 on responses to pervanadate

The effects of the calmodulin antagonist W-7 and the MLCK antagonist ML-7 on myogenic tone and responses to pervanadate are shown in Fig. 5. In the absence of pervanadate, W-7 (50 μM) significantly dilated the vessels to

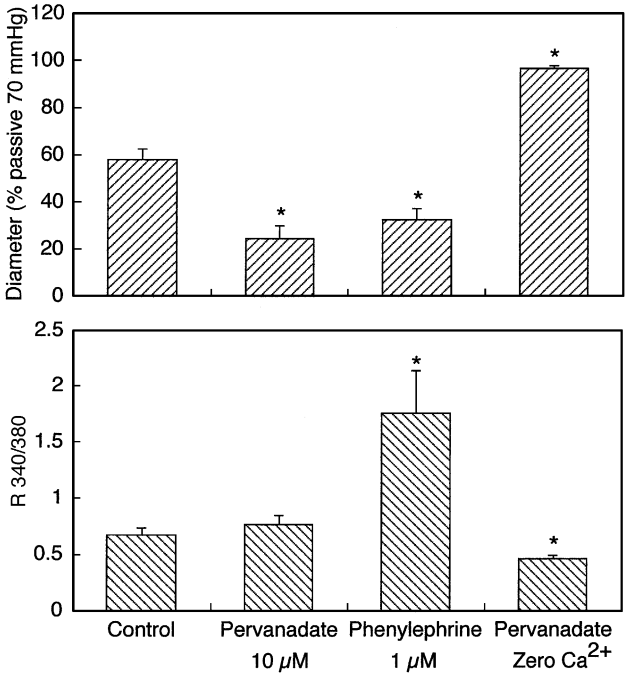

Fig. 4. Effect of pervanadate (10 μM) or phenylephrine (1 μM) on diameter (upper panel) and arteriolar wall [Ca^{2+}] (lower panel) of rat cremaster arterioles pressurized to 70 mmHg. Diameter is shown as a percentage of the maximum arteriolar diameter as determined in Ca^{2+}-free buffer. Arteriolar wall [Ca^{2+}] was measured using the Ca^{2+}-sensitive dye Fura-2 and is shown as the ratio of fluorescence of arterioles stimulated at 340 and 380 nm (R 340/380). Five experiments also examined the effect of 100 μM pervanadate on diameter and arteriolar wall [Ca^{2+}] in the absence of extracellular Ca^{2+}. Columns represent the mean ± S.E.M. * indicates a significant difference from the corresponding control value ($P<0.05$, ANOVA followed by Dunnett's test).

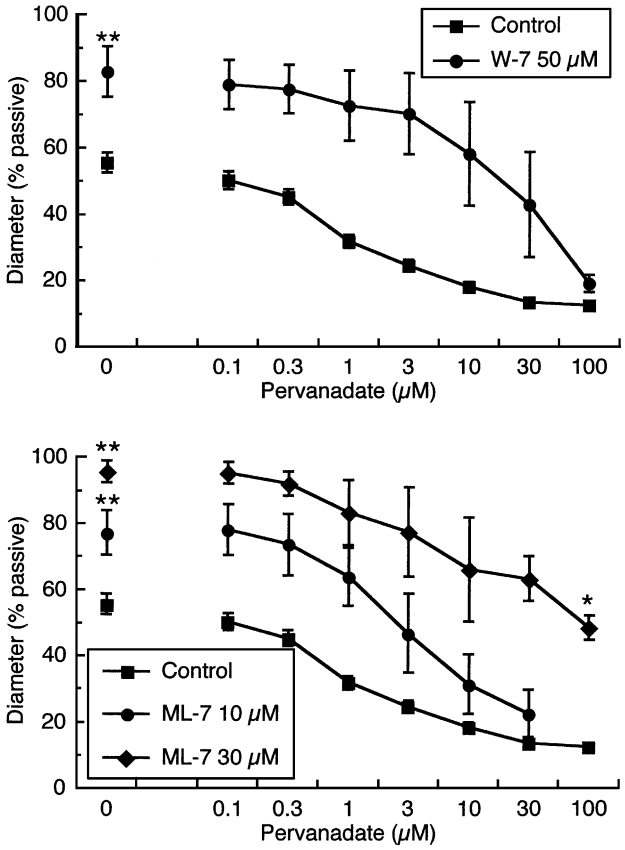

Fig. 5. Effects of W-7 (50 μM; upper panel) or ML-7 (10 or 30 μM; lower panel) on pervanadate-induced contractions of pressurized rat cremaster arterioles. Consecutive, cumulative concentration–response curves to pervanadate were constructed and the inhibitor, where present, was added to the vessel bathing solution 15 min before the construction of the second curve. Points represent the mean ± S.E.M. * indicates a significant reduction in the maximum contraction to pervanadate ($P<0.05$, ANOVA followed by Dunnett's test). ** indicates a significant effect of tyrphostin on the diameter of the vessels in the absence of pervanadate ($P<0.05$, Dunnett's test). Upper panel: ■ Control, $n=5$; ● W-7, $n=5$. Lower panel: ■ Control, $n=10$; ● ML-7 10 μM, $n=5$; ▲ ML-7 30 μM, $n=5$.

82.9 ± 7.6% of the maximal diameter, abolishing approximately 71% of the myogenic tone present at 70 mmHg in these vessels ($P<0.05$, Dunnett's test; Fig. 5). W-7 also inhibited the constrictor response of the arterioles to pervanadate; the pEC_{50} for pervanadate was reduced significantly ($P<0.05$, Table 1) while the maximum contraction to pervanadate was also slightly, but significantly reduced ($P<0.05$, Dunnett's test; Fig. 5).

The MLCK inhibitor ML-7 (10 or 30 μM) similarly dilated vessels with myogenic tone, the lower concentration of ML-7 (10 μM) abolished approximately 60% of myogenic tone while in the presence of 30 μM ML-7, 90% of myogenic tone was abolished at 70 mmHg (Fig. 5). The lower concentration of ML-7, while dilating the arteriole, did not significantly alter the constriction response to pervanadate, either in terms of the pEC_{50} (Table 1) or the maximum response (Fig. 5). In contrast, 30 μM ML-7

inhibited the contractile effect of pervanadate as assessed by these parameters (Table 1; Fig. 5).

4. Discussion

Contraction of smooth muscle caused by pervanadate is thought to be due to inhibition of tyrosine phosphatases and subsequently increased tyrosine phosphorylation of various tyrosine kinase substrates, many of which remain to be identified (Laniyonu et al., 1994; Di Salvo et al., 1997; Hughes and Wijetunge, 1998). Previous studies in vascular smooth muscle have suggested that an important component of pervanadate-induced contraction is increased Ca^{2+} influx through voltage-sensitive channels, especially L-type channels (Laniyonu et al., 1994; Wijetunge et al., 1998, Kimura et al., 2000). However, the findings of the present study suggest only a minor role for L-type VSCCs or indeed increase in intracellular [Ca^{2+}] (i.e. above basal levels) in pervanadate-induced constriction of pressurized rat cremaster muscle arterioles. The effects of pervanadate appear, however, to be dependent on both Ca^{2+} and flux through the Ca^{2+}/calmodulin/MLCK pathway as the abolition of Ca^{2+} from the bathing medium virtually abolished contraction while inhibition of calmodulin or MLCK significantly reduced pervanadate-induced constrictions.

Pervanadate-induced contractions were attenuated by the tyrosine kinase inhibitor tyrphostin 47, suggesting that the contraction was due to increased tyrosine phosphorylation. Consistent with this, our previous work showed that pervanadate increased phosphotyrosine levels in the rat cremaster arteriole, as measured by increased fluorescence intensity of arterioles incubated with a fluorophore-conjugated phosphotyrosine antibody. The increase in fluorescence intensity was also attenuated by tyrphostin 47 (Spurrell et al., 2000). Further, Western transfer techniques in combination with a monoclonal antibody for phosphotyrosine show a marked increase in tyrosine phosphorylation in pervanadate-stimulated arterioles (data not shown). In the present study and previous investigations, tyrphostin alone dilated the arterioles, suggesting a role for tyrosine kinases in maintaining pressure-induced myogenic tone (Spurrell et al., 2000). A study in rat cerebral arteries (Masumoto et al., 2000) reported similar findings. Mechanical stretch, a stimulus similar to the increased transmural pressure applied to cannulated blood vessels, increased protein tyrosine phosphorylation in strips of porcine carotid artery (Adam et al., 1995; Franklin et al., 1997) and sheep tracheal smooth muscle (Tang et al., 1999). Therefore, it seems likely that increased phosphotyrosine formation, presumably through increased tyrosine kinase activity plays a role in maintaining pressure-induced smooth muscle tone, although the precise mechanism through which this occurs is yet to be identified.

The voltage-sensitive Ca^{2+} channel (VSCC) inhibitors nifedipine and verapamil had varying effects on pervanadate-induced contractions. Verapamil was without effect, nifedipine failed to alter constriction at the lower concentration used, but did reduce contractions at a higher concentration (10 μM), by approximately 40%. These observations suggest little role for VSCCs in pervanadate-induced contractions of skeletal muscle arterioles. The overall effectiveness of Ca^{2+} channel blockade can be gauged by the fact that both Ca^{2+} channel inhibitors dilated the vessels significantly, as myogenic tone is dependent upon smooth muscle depolarization, activation of these Ca^{2+} channels and subsequent entry of extracellular Ca^{2+} (Meininger et al., 1991; Wesselman et al., 1996, Potocnik et al., 2000). Furthermore, in the concentrations used, both nifedipine and verapamil have been shown to greatly reduce or abolish K^+-induced contraction in this preparation (Potocnik et al., 2000), an effect also dependent on activation of voltage-sensitive Ca^{2+} channels (Bolton, 1979).

In contrast to these findings, Laniyonu et al. (1994) showed that 1 μM nifedipine reduced pervanadate contraction of rat aortic strips by 75%, although these investigators also noted that verapamil and another VSCC antagonist, diltiazem, were much less effective than nifedipine. In addition, pervanadate and other tyrosine phosphatase inhibitors were shown to increase current carried by VSCCs in isolated smooth muscle cells, an effect associated with increased tyrosine phosphorylation of the channel proteins (Wijetunge et al., 1998). Conceivably, this difference in regulation of VSCCs by tyrosine phosphorylation in vascular preparations may be due to an altered activation state of the channels. In non-contracted (non-myogenically contracted) smooth muscle strips or isolated cells, it is likely that VSCCs are at a low level of activation, whereas the same channels are more active in pressurized vessels, as the smooth muscle cell membrane is depolarized (Harder et al., 1987; Potocnik et al., 2000). In preparations where VSCCs are already activated, increased tyrosine phosphorylation with an agent such as pervanadate may be unable to cause a further increase in VSCC activity. However, this argument is not supported by our previous studies, in which tyrosine kinase inhibitors failed to reduce intracellular [Ca^{2+}] in pressurized arterioles (Spurrell et al., 2000). Possibly the contrasting effects of VSCC blockers on pervanadate-induced contractions in aorta and arterioles reflect differences in the channels themselves (Morita et al., 1999) and/or a difference in regulatory mechanisms.

The effects of pervanadate on intracellular [Ca^{2+}] in the arteriole were examined more directly using the Ca^{2+} indicator dye Fura-2. Pervanadate did not increase arteriolar wall Ca^{2+} significantly, in contrast to the α-adrenoceptor agonist phenylephrine which caused a rapid increase in arteriolar wall Ca^{2+} during contraction, including those contractions elicited in the presence of pervanadate (Spurrell et al., 2000). These findings are consistent with our previous studies, in which tyrosine kinase inhibitors genistein and tyrphostin 47 dilated pressurized cremaster arterioles without significantly reducing intracellular Ca^{2+} (Spurrell et al., 2000), suggesting that protein tyrosine phosphorylation

does not interact with Ca^{2+} mobilization pathways in this vessel.

Despite its comparative lack of effect on Ca^{2+} influx or intracellular [Ca^{2+}], pervanadate contractions were dependent on Ca^{2+} as complete removal of Ca^{2+} from the bathing medium (and the addition of the Ca^{2+} chelating agent EGTA) abolished the effect. Furthermore, it is likely that pervanadate-induced contractions of smooth muscle involve increased myosin light-chain phosphorylation through the Ca^{2+}/calmodulin/MLCK pathway. Consistent with this, the calmodulin inhibitor W-7 and the MLCK inhibitor ML-7 attenuated pervanadate-induced contractions. W-7 and ML-7 also caused dilation of arterioles with myogenic tone, to a similar extent as the VSCC inhibitors, but unlike the Ca^{2+} channel blockers W-7 and ML-7 also reduced the potency (pEC_{50}) of pervanadate. Under the conditions of the present study, it is likely that intracellular [Ca^{2+}] was increased to a level sufficient to cause activation of calmodulin and MLCK. Thus, our previous studies in the rat cremaster arteriole showed that elevation of intra-luminal pressure causes increased phosphorylation of the 20-kDa light-chain of myosin (LC_{20}), an effect not observed in the absence of extracellular Ca^{2+} (Zou et al., 1995, 2000).

The observation that tyrosine phosphatase inhibition could constrict the cremaster arteriole in the absence of an overt increase in intracellular Ca^{2+}, but with an apparent requirement for flux through the calmodulin/MLCK pathway and increased LC_{20} phosphorylation, suggests that increased myofilament Ca^{2+} sensitivity may be involved in this response. Some evidence exists to support a role for tyrosine kinases or increased tyrosine phosphorylation in vascular myofilament Ca^{2+} sensitization, such as that induced by carbachol and endothelin-1 (Sato et al., 2000) or rho p21 protein (Sasaki et al., 1998), although negative findings have also been reported for endothelin-1 (Ohanian et al., 1997; Evans et al., 1999). Further investigation is required to establish the role of myofilament Ca^{2+} sensitization in contractile responses to pervanadate.

In summary, pervanadate-induced contraction of pressurized rat cremaster arteriole was mediated by increased tyrosine phosphorylation. A relatively high concentration of the VSCC antagonist nifedipine partially reduced pervanadate contractions, whereas verapamil was without effect and pervanadate contractions occurred in the absence of an increase in intracellular [Ca^{2+}]. However, there is a role for Ca^{2+} in the contractile effect of pervanadate, along with calmodulin and MLCK. These observations suggest that pervanadate may have increased the Ca^{2+} sensitivity of the myofilaments, through a mechanism involving increased protein tyrosine phosphorylation. Given the apparent dependency of pervanadate constriction on a myosin light-chain phosphorylation mechanism, it is conceivable that inhibition of myosin phosphatase is involved as has been proposed for a number of contractile mechanisms (see Somlyo and Somlyo, 2000). However, the individual proteins undergoing tyrosine phosphorylation in response to pervanadate which may be involved in enhancing myofilament Ca^{2+} sensitivity have yet to be identified.

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References

Adam, L.T., Franklin, M.T., Raff, G.F., Hathaway, D.R., 1995. Activation of mitogen-activated protein kinase in porcine carotid arteries. Circ. Res. 76, 183–190.

Alcon, S., Camello, P.J., Garcia, L.J., Pozo, M.J., 2000. Activation of tyrosine kinase pathway by vanadate in gallbladder smooth muscle. Biochem. Pharmacol. 59, 1077–1089.

Bevan, A.P., Drake, P.G., Yale, J.F., Shaver, A., Posner, B.I., 1995. Peroxovanadium compounds: biological actions and mechanism of insulin-mimesis. Mol. Cell. Biochem. 153, 49–58.

Bolton, T.B., 1979. Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev. 59, 606–718.

Di Salvo, J., Gifford, D., Kokkinakis, A., 1989. ATP- and polyphosphate mediated stimulation of $pp60^{c\text{-}src}$ kinase activity in extracts from vascular smooth muscle. J. Biol. Chem. 264, 10773–10778.

Di Salvo, J., Steusloff, A., Semenchuk, L., Satoh, S., Kolquist, K., Pfitzer, G., 1993. Tyrosine kinase inhibitors suppress agonist-induced contractions in smooth muscle. Biochem. Biophys. Res. Commun. 190, 968–974.

Di Salvo, J., Pfitzer, G., Semenchuk, L.A., 1994. Protein tyrosine phosphorylation, cellular Ca^{2+}, and Ca^{2+} sensitivity for contraction of smooth muscle. Can. J. Physiol. Pharmacol. 72, 1434–1439.

Di Salvo, J., Nelson, S.R., Kaplan, N., 1997. Protein tyrosine phosphorylation in smooth muscle: a potential coupling mechanism between receptor activation and intracellular calcium. Proc. Soc. Exp. Biol. Med. 214, 285–301.

Elberg, G., Li, J., Leibovitch, A., Schechter, Y., 1995. Non-receptor cytosolic tyrosine kinases from various rat tissues. Biochim. Biophys. Acta 1269, 299–306.

Evans, A.M., Cobban, H.J., Nixon, G.F., 1999. ET(A) receptors are the primary mediators of myofilament calcium sensitization induced by ET-1 in rat pulmonary artery smooth muscle: a tyrosine kinase independent pathway. Br. J. Pharmacol. 127, 153–160.

Franklin, M.T., Wang, C.L.-A., Adam, L.P., 1997. Stretch-dependent activation and desensitization of mitogen-activated protein kinase in carotid arteries. Am. J. Physiol. 273, C1819–C1827.

Harder, D.R., Gilbert, R., Lombard, J.H., 1987. Vascular muscle cell depolarization and activation in renal arteries on elevation of transmural pressure. Am. J. Physiol. 253, F778–F781.

Hatakeyama, N., Mukhopadhyay, D., Goyal, K., Akbarali, H.I., 1996. Tyrosine kinase-dependent modulation of calcium entry in rabbit colonic muscularis mucosae. Am. J. Physiol. 270, C1780–C1789.

Heffetz, D., Bushkin, I., Dror, R., Zick, Y., 1990. The insulinomimetic agents H_2O_2 and vanadate stimulate protein tyrosine phosphorylation in intact cells. J. Biol. Chem. 265, 2896–2902.

Horowitz, A., Menice, C.B., Laporte, R., Morgan, K.G., 1996. Mechanisms of smooth muscle contraction. Physiol. Rev. 76, 967–1003.

Hughes, A.D., Wijetunge, S., 1998. Role of tyrosine phosphorylation in excitation–contraction coupling in vascular smooth muscle. Acta Physiol. Scand. 164, 457–469.

Kaplan, N., Di Salvo, J., 1996. Coupling between [$arginine^8$]–vasopressin-activated increases in protein tyrosine phosphorylation and cellular cal-

cium in A7r5 aortic smooth muscle cells. Arch. Biochem. Biophys. 326, 271–280.

Kimura, M., Obara, K., Sasase, T., Ishikawa, T., Tanabe, Y., Nakayama, K., 2000. Specific inhibition of stretch-induced increase in L-type calcium channel currents by herbimycin A in canine basilar arterial myocytes. Br. J. Pharmacol. 130, 923–931.

Laniyonu, A., Saifeddine, M., Ahmad, S., Hollenberg, M.D., 1994. Regulation of vascular and gastric smooth muscle contractility by pervanadate. Br. J. Pharmacol. 113, 403–410.

Masumoto, N., Tanabe, Y., Saito, M., Nakayama, K., 2000. Attenuation of pressure-induced myogenic contraction and tyrosine phosphorylation by fasudil, a cerebral vasodilator, in rat cerebral artery. Br. J. Pharmacol. 130, 219–230.

Meininger, G.A., Zaweija, D.C., Falcone, J.C., Hill, M.A., Davey, J.P., 1991. Calcium measurement in isolated arterioles during myogenic and agonist stimulation. Am. J. Physiol. 261, H950–H959.

Morita, H., Cousins, H., Onoue, H., Ito, Y., Inoue, R., 1999. Predominant distribution of nifedipine-insensitive, high voltage-activated Ca^{2+} channels in the terminal mesenteric artery of guinea pig. Circ. Res. 85, 596–605.

Ohanian, J., Ohanian, V., Shaw, L., Bruce, C., Heagerty, A.M., 1997. Involvement of tyrosine phosphorylation in endothelin-1-induced calcium-sensitization in rat small mesenteric arteries. Br. J. Pharmacol. 120, 653–661.

Posner, B.I., Faure, R., Burgess, J.W., Bevan, A.P., Lachance, D., Zhang-Sun, G., Fantus, I.G., Ng, J.B., Hall, D.A., Lum, B.S., 1994. Peroxovanadium compounds. A new class of potent phosphotyrosine phosphatase inhibitors which are insulin mimetics. J. Biol. Chem. 269, 4596–4604.

Potocnik, S.J., Murphy, T.V., Kotecha, N., Hill, M.A., 2000. Effect of mibefradil and nifedipine on arteriolar myogenic responsiveness and intracellular Ca^{2+}. Br. J. Pharmacol. 131, 1065–1072.

Sandirasegarane, L., Gopalakrishnan, V., 1995. Vanadate increases cytosolic free calcium in rat aortic smooth muscle cells. Life Sci. 56, 169–174.

Sasaki, M., Hattori, Y., Tomita, F., Moriishi, K., Kanno, M., Kohya, T., Oguma, K., Kitabatake, A., 1998. Tyrosine phosphorylation as a convergent pathway of heterotrimeric G protein and rho-protein-mediated Ca^{2+} sensitization of smooth muscle of rabbit mesenteric artery. Br. J. Pharmacol. 125, 1651–1660.

Sato, H., Hattori, Y., Sasaki, M., Tomita, F., Kohya, T., Kitabatake, A., Kanno, M., 2000. Agonist-dependent difference in the mechanisms involved in Ca^{2+} sensitization of smooth muscle of porcine coronary artery. J. Cardiovasc. Pharmacol. 35, 814–821.

Sim, S.S., Kim, C.J., 1998. The involvement of protein kinase C and tyrosine kinase in vanadate-induced contraction. Arch. Pharm. Res. 21, 315–319.

Somlyo, A.P., Somlyo, A.V., 2000. Signal transduction by G-proteins, rho-kinase and protein phosphorylation to smooth muscle and non-muscle myosin II. J. Physiol. 522, 177–185.

Spurrell, B.E., Murphy, T.V., Hill, M.A., 2000. Tyrosine phosphorylation modulates arteriolar tone but is not fundamental to the myogenic response. Am. J. Physiol. 278, H373–H382.

Tang, D., Mehta, D., Gunst, S.J., 1999. Mechanosensitive tyrosine phosphorylation of paxillin and focal adhesion kinase in tracheal smooth muscle. Am. J. Physiol. 276, C250–C258.

Wesselman, J.P.M., VanBavel, E., Pfaffendorf, M., Spaan, J.A.E., 1996. Voltage-operated calcium channels are essential for the myogenic responsiveness of cannulated rat small mesenteric arteries. J. Vasc. Res. 33, 32–41.

Wijetunge, S., Lymn, J.S., Hughes, A.D., 1998. Effect of inhibition of tyrosine phosphatases on voltage-operated calcium channel currents in rabbit isolated ear artery cells. Br. J. Pharmacol. 124, 307–316.

Zou, H., Ratz, P.H., Hill, M.A., 1995. Role of myosin phosphorylation and $[Ca^{2+}]$ in myogenic reactivity and arteriolar tone. Am. J. Physiol. 269, H1590–H1596.

Zou, H., Ratz, P.H., Hill, M.A., 2000. Temporal aspects of Ca^{2+} and myosin phosphorylation during myogenic and agonist-induced arteriolar constriction. J. Vasc. Res. 37, 556–567.