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Characterization of Vanadyl Sulfate Effect on Vascular Contraction: Roles of Calcium and Tyrosine Phosphorylation[1]

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ABSTRACT

In order to explore the mechanism of action of vanadyl sulfate ($VOSO_4$), previously described as an antidiabetic and antihypertensive agent, we have investigated the role of calcium and tyrosine phosphorylation in the contractile responses of rat aorta or skinned rabbit mesenteric artery rings. $VOSO_4$ induced a concentration-dependent contraction of aorta ($pD_2 = 3.2$), which was potentiated by endothelium removal ($pD_2 = 4.2$). After a first exposure to $VOSO_4$, no change in responsiveness was observed even though high vanadium concentrations had accumulated in the aortic tissue ($\approx 4 \times 10^{-3}$ M). $VOSO_4$ induced, in calcium-free medium, a significant response that, relative to contractions measured in Krebs-Henseleit buffer, was higher (36%) than norepinephrine (16%)-, arginine-vasopressin (8%)- or KCl (5%)-induced responses. 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8), an intracellular calcium release inhibitor, did not modify $VOSO_4$-induced response either in the presence or in the absence of ambient calcium. On skinned preparations, $VOSO_4$ antagonized Ca^{++}-induced contraction. The tyrosine kinase inhibitors tyrphostin 23 (T_{23}) and tyrphostin 47 (T_{47}) potentiated by 4- and 14-fold, respectively, the activity of $VOSO_4$, in contrast to the lack of effect of T_{47} on pervanadate-induced contraction. When phosphotyrosine content was revealed by Western blotting, $VOSO_4$ had no effect alone, but in the presence of T_{47}, it dramatically increased the phosphotyrosine content. This result contrasts again with PV-induced tyrosine phosphorylation, which was blocked by T_{47}. These data suggest that the signaling events involved in vascular effects of $VOSO_4$, although they depend little on calcium mobilization, are related to tyrosine phosphorylation, likewise through a pathway different from that of pervanadate.

The pharmacology of vanadium, a Group Vb transition metal, has been extensively investigated in the last decade, particularly in terms of its insulinomimetic properties (Brichard and Henquin, 1995; Cros *et al.*, 1992), and their possible therapeutic application in diabetes mellitus (Cam *et al.*, 1993; Goldfine *et al.*, 1995; Cohen *et al.*, 1995).

Compelling evidence had accumulated, showing differences in the cellular mechanism of action of the various vanadium species. Vanadate (V^{5+}) and vanadyl (V^{4+}) derivatives have *in vivo* antidiabetic properties correlated with an increase in insulin sensitivity, whereas their cellular mechanism, though it remains controversial, is thought to be independent of insulin receptor activation (Goldfine *et al.*, 1995). More recently developed peroxovanadium (pervanadate) derivatives were shown to be able to lower blood glucose in the insulin-dependent diabetic BB rat *in vivo* (Yale *et al.*, 1995), and their cellular mechanism seems to be directly linked to insulin receptor activation (Bevan *et al.*, 1995). The vanadate (V^{5+}) and vanadyl (V^{4+}) oxidation states also differ markedly in biochemical (Cantley and Aisen, 1979; Elberg *et al.*, 1994), pharmacological (Bhanot and McNeill, 1994; Boscolo *et al.*, 1994; Nakai *et al.*, 1995) and toxicologic (Llobet and Domingo, 1984) properties. In particular, vanadate derivatives were shown to have prohypertensive properties (Boscolo *et al.*, 1994), whereas vanadyl derivatives were shown to be antihypertensive agents (Bhanot and McNeill, 1994; Bhanot *et al.*, 1994).

Since 1980 (Ozaki and Urakawa, 1980) it has been known that vanadium salts are able to induce contraction of a variety of smooth muscles tissues, including vascular smooth muscle. Vanadate salts have been studied most, and their mechanism of action remains uncertain despite extensive investigations. The structural similarity between vanadate—but not vanadyl—and phosphate groups, as well as the inhibitory effect of vanadate on sarcoplasmic and endoplasmic calcium-ATPase (SERCA) (Raeymakers *et al.*, 1983), suggested the mobilization of intracellular calcium stores

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ABBREVIATIONS: AVP, [Arginine^8]-vasopressin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetra-acetic acid; N, nicardipine; NEPI, norepinephrine; $VOSO_4$, vanadyl sulfate; TMB-8, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride; $T_{1,23,47,63}$, tyrphostins 1, 23, 47, 63; PV, pervanadate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide.

(Sanchez-Ferrer *et al.*, 1988). Since the description of various isoforms of SERCA, however, it has recently been shown that vanadate has no effect on muscular isoforms (Lytton *et al.*, 1992). The increased level of tyrosine phosphorylation, typically described in the mechanism of the insulinomimetic effects of vanadium derivatives (Tamura *et al.*, 1984; Shechter *et al.*, 1995), was also found to be associated with the smooth muscle contractile activities of vanadate and PV (DiSalvo *et al.*, 1993; Laniyonu *et al.*, 1994), which are still unknown in the case of vanadyl.

The aim of the present study was to use isolated vascular preparations to characterize further the vascular properties of $VOSO_4$ and to study its mechanism of action. In particular, we examined the relative contributions of calcium and tyrosine phosphorylation to its activity. We describe the concentration-dependent $VOSO_4$-induced contraction in aortic tissue that is regulated by, but not dependent on, the presence of endothelium and that occurs in the absence of extracellular calcium or the presence of blockers for extracellular calcium entry and intracellular calcium mobilization. Surprisingly, the activity of $VOSO_4$, but not that of the potent tyrosine phosphatase inhibitor PV, was amplified by the tyrosine kinase inhibitors tyrphostins, concomitantly with elevated levels of tyrosine phosphorylation.

Materials and Methods

Contractile effect of $VOSO_4$ on rat isolated aorta. Thoracic aorta rings 3 mm long were was obtained from male Wistar rats (300–350 g) and suspended at 37°C in aerated (95% O_2, 5% CO_2) Krebs-Henseleit buffer (mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.0; $KH2PO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.1; pH 7.4. Isometric tension recording was performed in tissues equilibrated for 60 min under 1 g tension, washed with buffer every 15 min and then exposed to 40 mM KCl to test the viability and "sensitize" preparations for subsequent exposure to contractile agents. When indicated, a second KCl (40 mM)-induced response was recorded as "internal standard." $VOSO_4$ was used either as single concentrations (10^{-4} or 5×10^{-4} M) or as cumulative concentrations (1 to 1000 μM). Vanadium levels were determined by atomic absorption spectrophotometry (Mongold *et al.*, 1990).

Endothelium was removed by gentle rubbing of the internal lumina of aortic rings. The effect of endothelium removal was confirmed by the inability of ACh (10^{-5} M) to induce vasodilation after NEPI (6×10^{-7} M)-induced contraction.

Role of calcium in contractile activity of $VOSO_4$. The role of calcium was first assessed on rat thoracic aorta. KCl (40 mM), AVP (3×10^{-8} M) and NEPI (6×10^{-7} M) were used for comparison purposes. The amplitude of responses of these agonists was comparable to that of $VOSO_4$ (5×10^{-4} M). Consecutive exposures to the same agent yielded similar responses (not illustrated).

The effect of the calcium entry blocker N (0.01–1 μM) and that of the intracellular calcium liberation inhibitor TMB-8 (30 μM) (Chiou and Malagodi, 1975) were assessed as follows: The preparation was first exposed to the contractile agent (designated as control contraction) and was then washed and exposed to N or TMB-8 for 20 or 30 min, respectively. Finally, the tissue preparation was exposed to the contractile agent in the presence of antagonist.

To investigate the role of calcium-free buffer, contraction was measured after subsequent incubations of arterial rings for 45 min in calcium-free buffer and then for 15 min in the presence of 1 mM EDTA, followed by extensive washing in calcium-free buffer without EDTA.

The role of calcium was also assessed by using skinned rabbit mesenteric arteries prepared with saponin according to the method of Sunano *et al.* (1988). Briefly, mesenteric arteries were removed and cleaned of fat and fibrous tissue. Arterial vessel was cut into rings and placed in aerated (95% O_2, 5% CO_2) relaxing medium containing 130 mM KCl, 20 mM Tris-maleate, 5 mM Na_2ATP and 4 mM EGTA, pH 6.8, at 25°C. Preparations were equilibrated under 1 g tension, treated for 20 min with 50 μg/ml saponin and then washed for another 20 min. Thereafter, mesenteric arteries were submitted to cumulative concentrations of free Ca^{++} (obtained by adding $CaCl_2$ to the medium containing 4 mM EGTA) in the absence or presence of $VOSO_4$. Free Ca^{++} concentrations were calculated according to the method described by Fabiato and Fabiato (1979).

Role of tyrosine phosphorylation. The role of tyrosine phosphorylation in the contractile activity of Ca^{++} was assessed by using the tyrosine kinase inhibitors genistein, T_{47} (RG 50864) and T_{23} (RG 50810), as well as inactive tyrphostin derivatives (T_1 and T_{63}). The tyrosine kinase inhibitors were dissolved in dimethylsulfoxide (DMSO) and used at a concentration of 0.2 mM. DMSO alone had no contractile activity at the maximal concentration used (0.1%).

The tyrosine kinase inhibitors were added 10 min before the following contractile agents: $VOSO_4$ (10^{-4} M), KCl (40 mM), AVP (3×10^{-8} M), NEPI (5×10^{-7} M) and PV (10^{-4} M). PV had previously been described as a potent inhibitor of tyrosine phosphatases and thus an inducer of tyrosine phosphorylation (Shisheva and Shechter, 1993a). In addition, the effects of T_{47} were examined in preparations without endothelium and in calcium-free medium. Responses were expressed as percent of KCl-induced contraction, described above as the "internal standard" in the absence of inhibitors.

Investigations of phosphotyrosine content were conducted by Western blotting on 8-mg segments of rat aorta prepared for isometric tension recording after removal of endothelium. Aortic segments were exposed to $VOSO_4$ (10^{-4} or 5×10^{-4} M), PV (10^{-4} M) or the associations of T_{47} plus $VOSO_4$ (10^{-4} M) or PV (10^{-4} M) and frozen at maximal contraction. For control, aortic preparations were frozen under basal tension (1 g) in the presence or absence of T_{47}. Tissues were kept at −80°C until processed for Western blotting.

Pieces of aortic tissue were thawed, homogenized at 4°C in a Thomas tissue grinder with a Teflon pestle, directly solubilized in Laemmli buffer and boiled for 3 min. Insoluble material was discarded after microfuge centrifugation (18 000 × *g*), solubilized proteins were resolved by SDS-PAGE (7.5% acrylamide) under reducing conditions (100 mM dithiothreitol) and transferred to nitrocellulose paper. Nitrocellulose strips were incubated for 8 h at 4°C in blocking solution containing 20 mM, 150 mM NaCl, 0.01% (v/v) Tween 20 and 3% bovine serum albumin. The antiphosphotyrosine recombinant antibody RC20, coupled with horseradish peroxidase (HRPO), was incubated in the blocking buffer at 1:250 dilution for 2 h at 22°C, and further steps were followed for ECL detection as recommended by the manufacturer (Amersham). For the detection of phosphotyrosine, lysates of A431 cells stimulated by EGF were used as standards as recommended by Affinity Research Products Ltd. (Exeter, UK).

Materials. $VOSO_4 \cdot 5H_2O$ and other reagents of analytical or sequence grade were from Prolabo (Paris, France). The PV solution was prepared according to Pumiglia *et al.* (1992) by incubating one part of 500 mM H_2O_2 with five parts of 10 mM sodium orthovanadate for 10 min at 37°C immediately before use. This procedure induced the formation of a mixture of peroxovanadium complexes (Campbell *et al.*, 1989) and has been widely used in pharmacological studies showing differences between the properties of PV and those of vanadate (Bevan *et al.*, 1995). AVP, NEPI, N, saponin, phenylmethylsulphonyl fluoride (PMSF), aprotinin and leupeptin were from the Sigma Chemical Co. (St Louis, MO). TMB-8, genistein and the tyrphostins were from Biomol (Plymouth Meeting, PA).

The RC20 linked to HRPO was from Affinity Research Products Ltd. Bradford protein assay, molecular weight standards and all compounds for electrophoresis were from Bio-Rad Chemical Division (Richmond, CA). Nitrocellulose paper (BA85, 0.2 mm) was from Schleicher & Schuell (Keene, NH).

Data analysis and statistics. Results of vascular contraction were expressed as mean ± S.E.M. Half-maximal effective concentrations (EC_{50}) were calculated using a computer program for multiple iterations on the Hill equation. pD_2 values were determined as log $1/EC_{50}$. Statistical comparisons were done using Newman-Keuls' test or Student's t test for paired or unpaired data, as appropriate. A probability value of less than 5% was considered significant.

Results

$VOSO_4$-induced contraction in rat aorta. Typical isolated aorta responses after the addition of a single concentration of $VOSO_4$ are shown in figure 1, panel A. $VOSO_4$ induced maximal vasoconstriction after 5 to 10 min. In most cases, a slowly developing contraction was followed by a steep increase in tension.

The role of the endothelium in the contractile properties of $VOSO_4$ was assessed by comparing $VOSO_4$ cumulative concentration-response curves in the presence or absence of endothelium (fig. 1, panel B). pD_2 was significantly higher in the absence of endothelium (4.06 ± 0.19) than in its presence (3.19 ± 0.12).

$VOSO_4$-induced contraction was readily reversible on washing and was reproducible in the same preparation for two consecutive cumulative concentration curves or two single-concentration (5×10^{-4} M) responses (data not shown). Vanadium tissue concentration after completion of one concentration-response curve averaged 576 ± 132 μg/g ($n = 4$). After extensive washing (six times) and return to baseline, it was still 190 ± 20 μg/g. These data indicate that vanadium accumulates in aortic segments at concentrations 10 times higher than in the buffer and that significant amounts of vanadium ($\approx 4 \times 10^{-3}$ M) remain stored in tissues after washing without significantly affecting further $VOSO_4$-induced responses.

Role of calcium. The influence of various concentrations of the calcium entry blocker N (0.01 to 1 μM) on $VOSO_4$-, AVP-, NEPI- and KCl-induced contractions is shown in figure 2. The inhibitory activity of N was concentration-dependent. The order of potency of N to inhibit contractile agents was AVP ≥ KCl >> $VOSO_4$ > NEPI.

In the absence of extracellular calcium, $VOSO_4$ at 5×10^{-4} M induced a slowly developing contraction with a maximum, after 20 to 30 min, reaching ~36% of the control contraction in standard buffer (not illustrated). This value was significantly higher than those obtained with KCl, AVP or NEPI (fig. 3).

The effect of the intracellular calcium liberation inhibitor TMB-8 on the activity of various contractile agents was measured in standard or calcium-free buffer (fig. 3). In standard

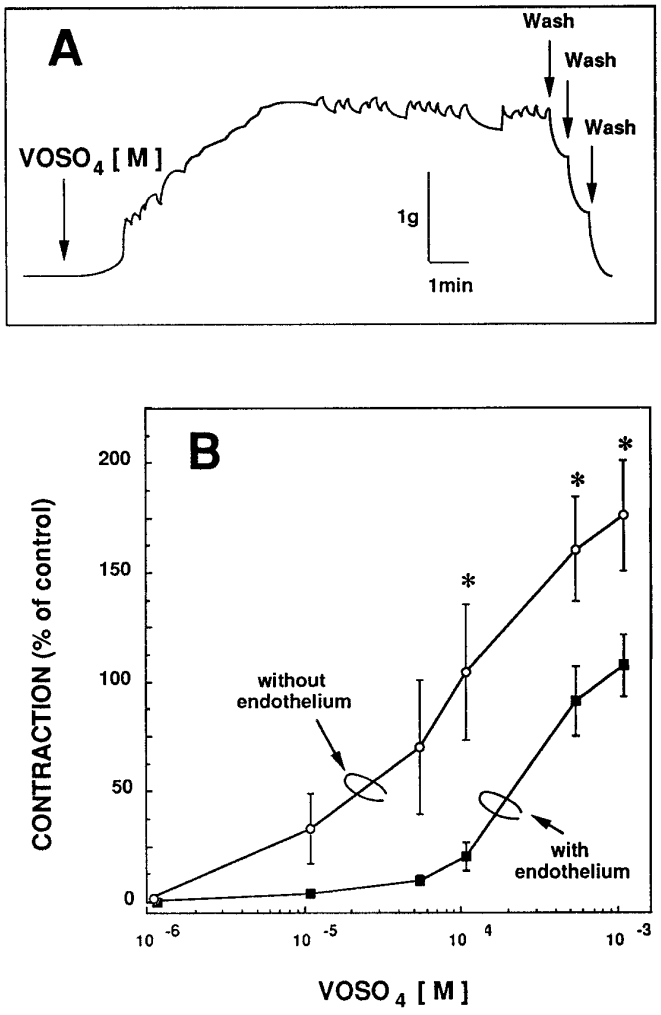

Fig. 1. $VOSO_4$-induced contraction on rat aorta. A) Typical tracing of aorta isometric contraction obtained at 5×10^{-4} M $VOSO_4$ in standard Krebs-Henseleit buffer as described in "Materials and Methods." B) Cumulative concentration-response curves of rat aorta contraction obtained with endothelium or after removal of endothelium. Contraction is expressed as percent of KCl (40 mM)-induced contraction (control). Points represent the mean ($n = 6$) and bars the S.E.M. Statistically significant differences are indicated by * ($P \leq .05$).

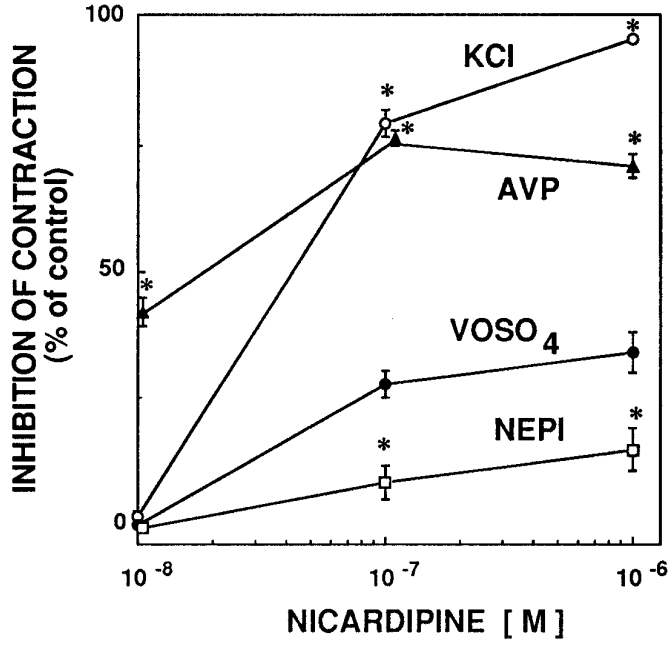

Fig. 2. Effect of nicardipine on the contractile responses of isolated rat aorta to various contractile agents. Isometric tension measurement of isolated segments of rat aorta was performed in standard Krebs-Henseleit buffer as described in "Materials and Methods." The effect of various concentrations of nicardipine on the contractile responses to $VOSO_4$ (5×10^{-4} M), KCl (40 mM), AVP (3×10^{-8} M) and NEPI (6×10^{-7} M) is expressed as percent of the control responses measured on the same preparations in the absence of nicardipine. Points represent the mean ± S.E.M. ($n = 8$). Statistically significant differences as compared with the effect of $VOSO_4$ are indicated by * ($P < .05$).

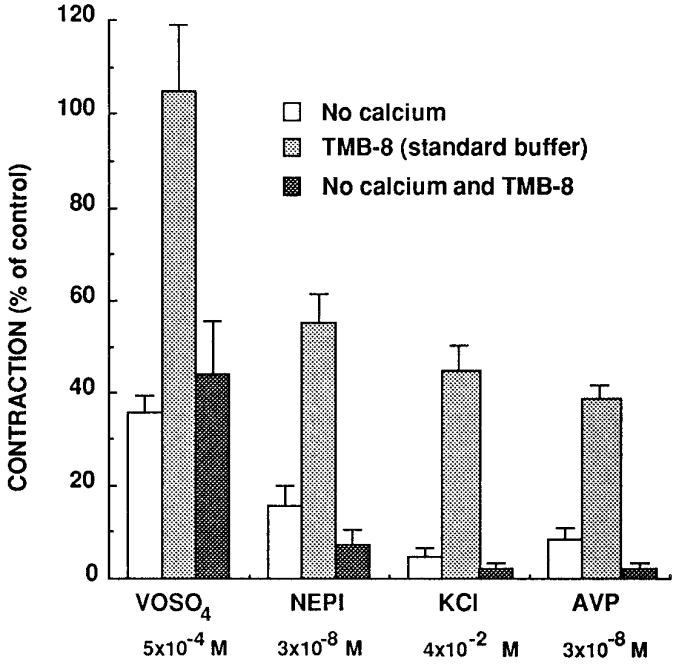

Fig. 3. Effects of calcium-free buffer and TMB-8 on the contractile responses of isolated aorta induced by various agents. Isometric tension responses of isolated rat aortic segments to $VOSO_4$ (5×10^{-4} M), KCl (40 mM), AVP (3×10^{-8} M) and NEPI (6×10^{-7} M) were measured in standard and in calcium-free buffer as described in "Materials and Methods," in the absence or presence of TMB-8 (30 μM). Results are expressed as percent of the control responses obtained in standard buffer on the same preparations. Values are means ± S.E.M. of 6 to 12 experiments. Results obtained with $VOSO_4$ are significantly different ($P \leq .05$) from those obtained with the other contractile agents in all cases.

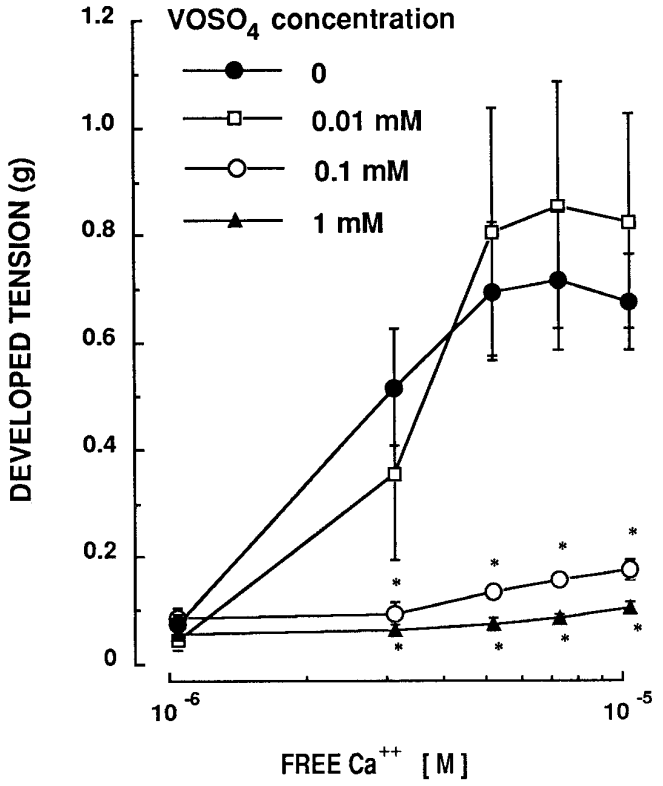

Fig. 4. Effect of Ca^{++} on rabbit skinned mesenteric arteries measured in the absence or presence of various concentrations of $VOSO_4$. Segments of rabbit mesenteric arteries were mounted for isometric tension measurement and skinned with saponin as described in "Materials and Methods." Free Ca^{++} concentrations were obtained by adding known amounts of $CaCl_2$ in the presence of 4 mM EGTA. Results are expressed as grams of developed tension. Values are means ± S.E.M. of 5 to 10 experiments. * indicates a significant difference ($P \leq .05$) compared with the contraction measured in the absence of $VOSO_4$.

buffer, responses to KCl, AVP and NEPI were significantly inhibited by TMB-8. In calcium-free buffer, the remaining fraction of NEPI-induced response was further reduced by TMB-8. By contrast, the effect of $VOSO_4$-induced contraction measured in standard or calcium-free buffer remained unchanged in the presence of TMB-8.

The possible relationship between calcium and $VOSO_4$ was studied on skinned vessels under conditions where intracellular Ca^{++} concentrations could be experimentally controlled. No contractile response to $VOSO_4$ could be obtained in the absence of calcium (data not shown). Figure 4 illustrates the effect of cumulated free Ca^{++} concentrations on the contraction measured in the absence or presence of three concentrations of $VOSO_4$. At 10^{-4} M and 10^{-3} M $VOSO_4$, Ca^{++}-induced responses were dramatically reduced. The same concentration-dependent inhibitory effect of $VOSO_4$ on Ca^{++}-induced contraction was also observed when $VOSO_4$ was added *after* the vessel had been maximally contracted with 10^{-5} M Ca^{++} (not illustrated).

Role of tyrosine phosphorylation. The influence of the tyrosine kinase inhibitors tyrphostins and genistein on the contractile activity of $VOSO_4$ is shown in figure 5. Genistein inhibited $VOSO_4$-induced contraction, but the effects of the tyrphostins varied according to the compound used (Gazit *et al.*, 1989). According to Gazit *et al.* (1989), tyrphostins T_1 and T_{63}, with $IC_{50} > 1200$ μM in the ability to inhibit EGF receptor tyrosine kinase, were designated as "inactive." Whereas the "inactive" tyrphostins T_1 and T_{63} had no significant effect on contraction, T_{47} ($IC_{50} = 2.4$ μM) dramatically (14-fold) potentiated the activity of $VOSO_4$. T_{23} ($IC_{50} = 35$ μM), a milder tyrosine kinase inhibitor than T_{47}, moderately (4-fold) potentiated the activity of $VOSO_4$. In contrast to its amplification effect on $VOSO_4$-induced contraction, T_{47} either had no effect on or inhibited the activity of KCl, NAD and AVP, whereas genistein was inhibitory in all cases (data not shown).

T_{47}-induced potentiation of $VOSO_4$ was still present after endothelium removal and in calcium-free buffer. Indeed, in the absence of endothelium, responses to 0.1 mM $VOSO_4$ were 26 ± 4% and 151 ± 2% of the KCl-induced response, in the absence and presence of T_{47}, respectively. In the absence of calcium, responses were 4 ± 1% and 53.9 ± 10%, in the absence and presence of T_{47}, respectively.

For comparison purposes, we also measured the effect of PV in the presence and absence of T_{47}. PV is a more specific tyrosine phosphatase inhibitor than vanadate or vanadyl derivatives and has previously been shown to induce high levels of tyrosine phosphorylation in smooth muscle preparations (Laniyonu *et al.*, 1994). The amplitude of contraction induced by 0.1 mM PV (19.2 ± 1.2%, $n = 6$) was higher than that induced with the same concentration of $VOSO_4$ (11.8 ± 1.7%, $n = 6$). However, no significant change in PV-induced contraction was observed in the presence of T_{47} (18.4 ± 1.3% of KCl-induced response, $n = 6$).

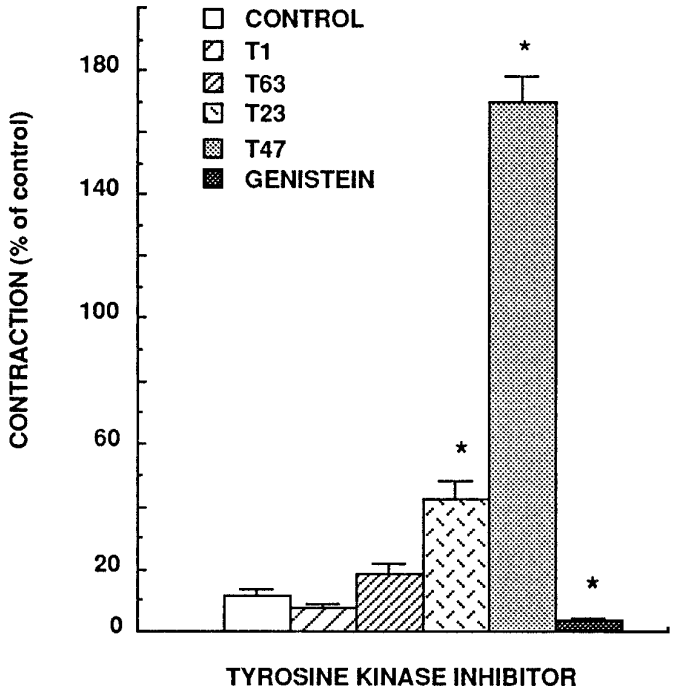

Fig. 5. Effect of various tyrosine kinase inhibitors on $VOSO_4$-induced contraction in rat aorta. Isometric tension measurement of isolated segments of rat aorta was performed in standard Krebs-Henseleit buffer as described in "Materials and Methods." The effect of various tyrosine kinase inhibitors (0.2 mM) on the contractile responses to $VOSO_4$ (10^{-4} M) is expressed as percent of the contraction induced by 40 mM KCl in the absence of tyrosine kinase inhibitor (control). Means values of 6 to 9 experiments are shown; vertical lines indicate S.E.M. * indicates a significant difference compared with the contraction obtained with $VOSO_4$ in the absence of inhibitors.

Tyrosine phosphorylation was determined by Western blotting (fig. 6) on endothelium-deprived aortic segments. Neither T_{47} nor $VOSO_4$ produced any apparent change in phosphotyrosine levels. However, the combination of T_{47} and $VOSO_4$ not only enhanced the $VOSO_4$-induced contractile response (fig. 5) but also produced a major increase in phosphotyrosyl content. Unlike $VOSO_4$ alone, PV alone increased the level of tyrosine phosphorylation. Although T_{47} had no effect on pervanade-induced contractile response, it inhibited the PV-induced increase in tyrosine phosphorylation.

Four major protein bands were intensively phosphorylated in the presence of PV or the association $VOSO_4$ and T_{47}, with apparent molecular weights of 200, 90, 70 and 30 kDa. Interestingly, only the protein band of 90 kDa was intensively phosphorylated by PV but not by the $VOSO_4$ and T_{47} association.

Discussion

Although the vasoconstricting properties of vanadium salts were known, the vascular properties of the vanadyl (V^{4+}) form of vanadium (V), recently described as antihypertensive (Bhanot and McNeill, 1994; Bhanot *et al.*, 1994), had not been specifically studied and characterized. In the present study, we showed that $VOSO_4$ produced a concentration-dependent vasoconstriction that was fully repeatable on the same preparation even in the presence of high vanadium tissue levels. Vasoconstriction 1) was not endothelium-dependent, 2) was present, although reduced in amplitude, in the absence of extracellular calcium and the presence of blockers of extracellular calcium entry or intracellular calcium mobilization and 3) was absent in skinned preparations. Unlike the response to the potent tyrosine phosphatase inhibitor PV, $VOSO_4$-induced vasoconstriction was amplified by tyrphostins. The amplification was associated with high levels of tyrosine phosphorylation.

$VOSO_4$ provoked vasoconstriction at concentrations (10–1000 μM) similar to those previously described with vanadate using the same experimental model (Shimada *et al.*, 1986; Laniyonu *et al.*, 1994; St-Louis *et al.*, 1995). These concentrations appear high compared with those used for known receptor-specific agonists, such as NEPI and AVP (1 pM–1 μM), but are lower than those used for KCl (10–100 mM), a known depolarizing agent. However, we recently showed that $VOSO_4$ was able to contract isolated smooth muscle cells at subnanomolar concentrations (Soulié *et al.*, 1996), while active concentrations of KCl remained within

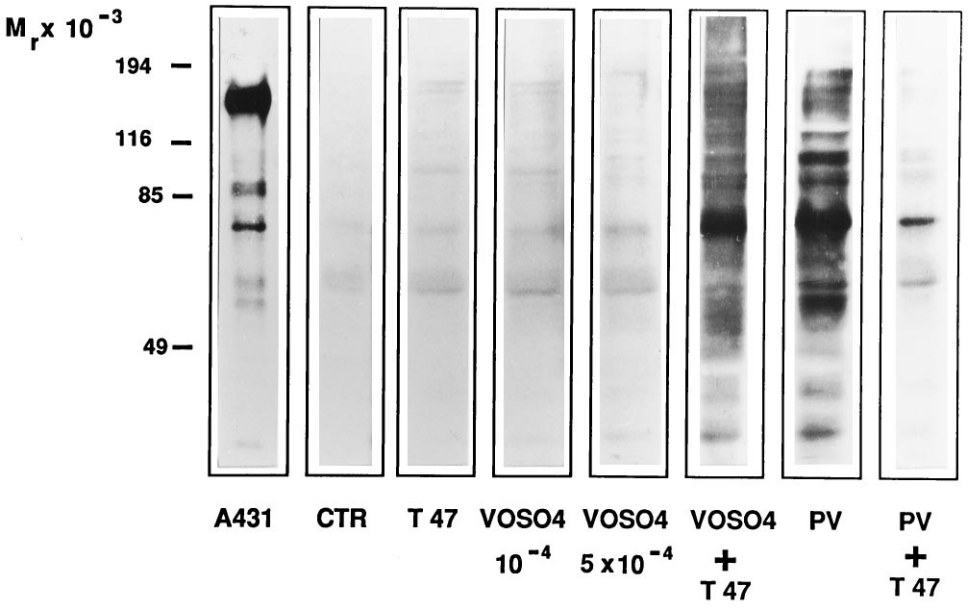

Fig. 6. Identification by Western blotting of phosphotyrosine content of aortic tissue under various conditions. Samples were prepared as described in "Materials and Methods" and resolved in SDS-PAGE. Immunoblotting was performed with RC20 antibodies and revealed by ECL detection buffers. First and second lanes correspond to A431 cell lysate and control aortic tissue without stimulation, respectively. The following lanes correspond to T_{47} (0.2 mM) alone, $VOSO_4$ (0.1 mM) alone, $VOSO_4$ (0.5 mM) alone, T_{47} (0.1 mM) and $VOSO_4$ (0.1 mM), pervanadate alone (10^{-4} M) and pervanadate (0.1 mM) and T_{47} (0.2 mM), respectively.

the 10 mM range (G. Cros, personal communication). These data indicate that $VOSO_4$ can contract smooth muscle cells at concentrations close to physiological vanadium levels. Considering the capacities of vanadium to bind to tissues, it is possible that accessibility to the reactive site is a major factor determining the active concentration of $VOSO_4$ in a given preparation.

In addition to yielding previously described vanadate effects, our studies also indicated that $VOSO_4$-induced vasoconstriction was fully repeatable on the same aortic segment, both at single and multiple cumulative concentrations. A recent study indicated that after a first exposure to PV, the aorta became unresponsive for more than 2 h not only to a second exposure to PV but also to other contractile agents (such as NEPI and KCl) (Laniyonu *et al.*, 1994). These data suggest that $VOSO_4$ and PV have different mechanisms of action.

$VOSO_4$-induced vasoconstriction was amplified by endothelium removal. In our study, the same amplification was also obtained in the presence of methylene blue, a well-known inhibitor of guanylyl cyclase (not illustrated), which clearly indicates that $VOSO_4$ vascular activity was not dependent on the presence of endothelium. This effect is probably modulated by endothelium relaxing factors rather than by liberation of vasoconstrictive mediators. Although Chung *et al.* (1992) described an endothelium-dependent relaxation factor induced by vanadate when aorta was fully contracted with NEPI, no significant relaxing effect was obtained with $VOSO_4$ under the same conditions (data not shown).

On the basis of *in vitro* experiments showing that vanadate (analogous to phosphate) was able to inhibit calcium ATPases (Raeymaekers *et al.*, 1983; Aureliano and Madeira, 1994), it has been widely accepted that inhibition of Ca^{++}-ATPase and the consequent intracellular calcium release are responsible for vanadate-induced contraction of smooth muscle (Sanchez-Ferrer *et al.*, 1988; Ozaki and Urakawa, 1980).

Because compelling studies have noted differences in pharmacological properties between vanadyl and vanadate salts, the role of calcium in the mechanism of action of $VOSO_4$ should be re-assessed.

Our results indicate that extracellular calcium influx plays a significant role in determining the amplitude of $VOSO_4$-induced contraction. Indeed, in the presence of the voltage-dependent slow Ca^{++} channel blocker N and in the absence of extracellular calcium, the $VOSO_4$-induced response was reduced to 70% and 35% of control, respectively. Thus, as is true of vanadate, $VOSO_4$ contractile activity in vascular tissue is not totally dependent on extracellular calcium, which suggests that calcium influx is not the initiating event of contraction.

Intracellular calcium release is unlikely to play a role for the following reasons: 1) In calcium-free buffer, a higher proportion of the contraction was conserved for $VOSO_4$ (35%) than for any other agent, including NEPI (17% of control). Because NEPI activity is dependent on calcium mobilization from intracellular stores followed by calcium entry from extracellular space (Kowarski *et al.*, 1985), comparison of the inhibiting effects of N and calcium-free buffer on NEPI-induced contraction (16% and 83% of control, respectively) indicates that a major proportion of intracellular mobilizable calcium is absent in our "calcium-free" experimental conditions. 2) TMB-8, a known intracellular calcium mobilization inhibitor, did not modify $VOSO_4$-induced contraction, either in the presence or in the absence of extracellular calcium, whereas it did inhibit NEPI-induced contraction. 3) Recent data (Lytton *et al.*, 1992) have shown that though all sarcoplasmic or endoplasmic Ca^{++}-ATPases (SERCA) isoforms are inhibited by the Ca^{++}-ATPase inhibitor thapsigargin, only the SERCA3 isoform is inhibited by vanadate. However, SERCA3 is not expressed in muscle, and other hypotheses must be considered.

It is also possible that the vanadyl ion directly activates the contractile machinery or sensitizes it to the effect of intracellular calcium. Experiments using skinned rabbit mesenteric arteries—the procedure is unsuccessful in rat aorta—showed that, much like vanadate (Nayler and Sparrow, 1983; Sunano *et al.*, 1988), $VOSO_4$ not only had no effect in the absence of calcium but also inhibited calcium-induced contractions at concentrations higher than 10 μM. Therefore, direct activation or sensitizing is unlikely.

Another hypothesis compatible with our results is that $VOSO_4$ initiates at the cellular membrane level a cascade of events leading to sensitization to the effects of intracellular calcium and, secondarily, to the entry of extracellular calcium. Such indirect sensitization would be impaired by saponin in our experimental conditions in which agonist-induced contraction is abolished (Itoh *et al.*, 1983). In addition, although the aortic tissue accumulated high amounts of vanadium ($\approx$4 mM) after a first exposure to $VOSO_4$, it was still able to respond to a low extracellular concentration of $VOSO_4$ (0.1 mM), which further suggests that the "initiating event" of contraction is located at the membrane level. A possible candidate for that "initiating event" is phospholipase D (PLD) activation. Indeed, PLD can be activated by an increase in tyrosine phosphorylation (Bourgoin and Grinstein, 1992), and an increase in tyrosine phosphorylation is involved in the mechanism of the insulinomimetic activity of vanadium salts (Brichard and Henquin, 1995). Activation of PLD induces the formation of phosphatidic acid and diacylglycerol, which could sensitize the contractile machinery to calcium without the elevation of intracellular calcium levels. In our study, the response to $VOSO_4$ was partially blocked by the PLD inhibitor butanol in the presence or absence of calcium (not illustrated), which suggests the possible participation of PLD in the $VOSO_4$-induced response.

Although the initiating event of contraction was not determined in the present study, the possible role of a tyrosine phosphorylation cascade in the regulation of $VOSO_4$-induced contraction was explored. Indeed, various studies have shown that tyrosine kinase inhibitors are able to antagonize the smooth muscle contractile activities of vanadate (Di Salvo *et al.*, 1994), PV (Laniyonu *et al.*, 1994) and even various agonists such as angiotensin II (Marrero *et al.*, 1994) and NEPI (Toma *et al.*, 1995). These results indicate that tyrosine phosphorylation plays a major role in the regulation of smooth muscle contraction (Hollenberg, 1994). Although the nonspecific tyrosine kinase inhibitor genistein, which blocks ATP binding to the enzyme, partially inhibited $VOSO_4$-, KCl-, AVP- and NEPI-induced contractions, the tyrphostins, which are analogous to phosphorylated tyrosine, dramatically amplified $VOSO_4$-induced vasoconstriction. Their potency was correlated with the ability to antagonize EGF (or PDGF)-induced tyrosine phosphorylation (Gazit *et al.*, 1989). These events occurred both in the presence and in

the absence of extracellular calcium and were not dependent on the presence of endothelium. Furthermore, though no change in the degree of tyrosine phosphorylation was apparent with $VOSO_4$ or T_{47}, a major phosphorylation occurred in the presence of both of them. Unlike $VOSO_4$, PV, a potent tyrosine phosphatase inhibitor, induced an increase in tyrosine phosphorylation that was prevented by T_{47}, whereas T_{47} had no influence on PV-induced contraction, as previously shown by Laniyonu *et al.* (1994).

These data indicate that the correlation between the contractile process and the degree of tyrosine phosphorylation is still unclear, even with compounds known to act on the level of tyrosine phosphorylation, such as PV. It is surprising that a tyrosine kinase inhibitor, which was able to inhibit the increase of tyrosine phosphorylation induced by PV in our experimental conditions, amplifies both $VOSO_4$-induced contraction and tyrosine phosphorylation. These data indicate either some additional unknown effect of tyrphostins or a complex regulatory process in the tyrosine phosphorylation cascade.

In addition, our data confirmed the differences previously noted among vanadium salts in their cellular mechanisms of action. Indeed, the insulinomimetic activity of PV has been related to the phosphosphorylation of insulin receptor (IR) by inhibition of the IR tyrosine phosphatase (Shisheva and Shechter, 1993a), whereas vanadyl and vanadate are thought to act on the postreceptor cascade (Shisheva and Shechter, 1993b). Contrary to what was recently shown in stretched or pharmacologically stimulated coronary arteries (Adam *et al.*, 1995), the activity of mitogen-activated protein kinase does not seem to be associated with $VOSO_4$-induced contraction, because no protein band was detected at 45 kDa. It is interesting to note, however, that among the three substrates (205, 116 and 86 kDa) shown by Di Salvo *et al.* (1993) to be tyrosine-phosphorylated during guinea pig taenia coli exposure to vanadate, two of them were also detected in our experimental conditions after exposure to PV (90 and 200 kDa) or to $VOSO_4$ and T_{47} (200 kDa). Further characterization and identification of these bands may yield new insights into the role of tyrosine phosphorylation in the regulation of smooth muscle contraction.

It was recently shown in rat adipocytes that vanadate was able to stimulate *in vitro* a staurosporine-sensitive cytosolic tyrosine kinase that might be involved in its insulinomimetic activity (Shisheva and Shechter 1993b; Shechter *et al.*, 1995). Preliminary results obtained in our laboratory on vascular tissue indicated that staurosporine had no effect on $VOSO_4$ in the absence of calcium, while blocking the amplification by T_{47} (data not shown). Further studies are necessary to establish whether such a tyrosine kinase is involved in the regulation of vascular smooth muscle contractility.

Finally, the question of why a vasoconstricting compound has antihypertensive properties should be asked. When used *in vitro*, vanadium may have different properties or even properties apparently opposite to those it exhibits during chronic *in vivo* studies, as we recently showed for insulin secretion (Cadène *et al.*, 1996). Another possibility is that although $VOSO_4$ induces vasoconstriction *in vitro*, chronic $VOSO_4$ treatment lowers insulin resistance—a known prohypertensive factor—and subsequently corrects hypertension (Bhanot *et al.*, 1995).

In summary, our results indicate that the vascular activity of $VOSO_4$, 1) is regulated by, but not dependent on, the presence of endothelium, 2) occurs in the absence of extracellular calcium and in the presence of an intracellular calcium mobilization blocker and 3) is amplified by the tyrosine kinase inhibitors tyrphostins (amplification is associated with high levels of tyrosine phosphorylation). As is true of its insulinomimetic activity, the cellular mechanism of the vascular activity of vanadyl appears different from that of PV. Further studies will determine the initiating event of $VOSO_4$-induced contraction. The surprising amplification of both $VOSO_4$-induced vascular response and tyrosine phosphorylation by a relatively specific growth factor tyrosine kinase inhibitor may be of interest for future studies on the regulation of tyrosine phosphorylation cascade and its role in the regulation of vascular contractility.

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