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Regulation of vascular and gastric smooth muscle contractility by pervanadate

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1 The contractile actions of vanadate (VO_4) and pervanadate (PV, peroxide(s) of vanadate) were studied in rat gastric longitudinal muscle strips and in aortic rings. The roles of extracellular sodium and calcium were evaluated and the potential effects of nerve-released agonists were considered. The possibility that these responses were due to the potentiation of tyrosine kinase activity, as a result of PV-mediated tyrosine phosphatase inhibition was explored with the use of tyrosine kinase inhibitors (genistein, tyrphostin) and by Western blot analysis of phosphotyrosyl proteins in PV-treated tissues. The ability of PV to mimic the action of the tyrosine kinase receptor-associated agonist, epidermal growth factor-urogastrone (EGF-Uro), in the gastric preparation was also studied.

2 PV caused concentration-dependent contractions in both gastric and aorta-derived tissues, with a potency that was 1 to 2 orders of magnitude greater than that of VO_4.

3 Although repeated exposure of gastric and aortic tissues to a fixed concentration of VO_4 caused reproducible contractions in both tissues, repeated exposure of gastric tissue to PV caused an increased contractile response plateauing after 3 exposures. In contrast, a single exposure of aortic tissue to PV (20 μM) caused a prolonged desensitization of the tissue to the subsequent contractile actions of PV or other agonists.

4 The contractile responses to PV were unaffected in both preparations by tetrodotoxin, atropine, yohimbine and phenoxybenzamine; and in the aortic preparation, the responses to VO_4 and PV were the same in the presence or absence of a functional endothelium.

5 PV-induced contractions in both tissues were observed in the absence of extracellular sodium but required extracellular calcium and were attenuated by 1 μM nifedipine.

6 In the gastric preparation, the characteristics of the contractile actions of PV paralleled those of EGF-Uro in terms of (1) inhibition by genistein, (2) inhibition by indomethacin and (3) a requirement for extracellular calcium. These response characteristics differed from those of other contractile agonists such as carbachol.

7 In both the gastric and aortic preparations genistein was able to inhibit PV-induced contractions selectively without causing comparable inhibition of KCl-induced contractions. Tyrphostin (AG18) also selectively blocked PV-induced contractions in the gastric, but not in the aortic preparation.

8 In both the gastric and aortic tissue, in step with an increased contractile response, PV caused increases in tissue phosphotyrosyl protein content, as detected by Western blot analysis using a monoclonal antiphosphotyrosine antibody; the increases in phosphotyrosyl protein content were reduced when tissues were treated with PV at the same time as a tyrosine kinase inhibitor.

9 PV, at sub-contractile concentrations, potentiated the contractile action of angiotensin II in both the gastric and aorta tissue.

10 We conclude that the growth factor-mimetic agent, PV, is a much more potent contractile agonist than VO_4 in both vascular and gastric smooth muscle tissue. PV can cause enhanced tissue phosphotyrosyl protein content most likely via the inhibition of tissue protein tyrosine phosphatases. The contractile actions of PV, which require extracelullar calcium and are independent of extracellular sodium, would appear not to be due either to Na^+/Ca^{2+} exchange, promoted by Na^+/K^+-ATPase inhibition or to the inhibition of Ca^{2+}-ATPase and might be best explained by the ability of PV, via tyrosine phosphatase inhibition, to potentiate a tyrosine kinase pathway linked to calcium entry and to the contractile process.

Keywords: Rat aorta; gastric smooth muscle; pervanadate; vanadate; tyrosine kinase; tyrosine phosphatase

Introduction

The role for cellular tyrosine kinases, tyrosine phosphatases and their substrates is most often thought of in the context of cell cycle control and the regulation of cell growth. These processes usually occur over a time course of several hours. Nonetheless, it has become evident over the past 6 to 8 years that so-called 'growth factors', such as epidermal growth factor-urogastrone (EGF-Uro) or platelet-derived growth factor, that act via tyrosine kinase receptor-mediated signal pathways, can acutely regulate vascular and gastric smooth muscle tension (summarized by Berk & Alexander, 1989; Hollenberg, 1994). Data have also been obtained to indicate that tyrosine kinase pathways may also be involved in the regulation of smooth muscle tension by agonists such as angiotensin II, that act via G-protein-coupled receptors (Saifeddine *et al.*, 1992; Yang *et al.*, 1993; Di Salvo *et al.*, 1993b). If the activation of a tyrosine kinase pathway by certain agonists can increase smooth muscle contractility, it can be hypothesized that cellular tyrosine phosphatase activity may act as a counter-regulatory factor to attenuate the actions of such agents. To explore this hypothesis, we considered the use of the insulin-mimetic agent, vanadyl

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hydroperoxide (peroxovanadate or pervanadate, PV), that acts in adipocytes (Kadota *et al.*, 1987), T-lymphocytes (O'Shea *et al.*, 1992), granulocytes (Bourgoin & Grinstein, 1992) or platelets (Blake *et al.*, 1993) by augmenting tyrosine kinase activity, presumably due to the inhibition of tyrosine phosphatase (Grinstein *et al.*, 1990; Heffetz *et al.*, 1990; Faure *et al.*, 1992) rather than via the inhibition of Na^+/K^+-ATPase (Dubyak & Kleinzeller, 1980). PV, which is much more potent than vanadate (VO_4) itself (Kadota *et al.*, 1987; Fantus *et al.*, 1989), is believed to be reduced intracellularly to the biologically active insulin-mimetic species. Prior to our study, the effects of PV in smooth muscle systems had not yet been explored.

A number of previous studies have examined the actions of VO_4 in a variety of vascular and non-vascular smooth muscle preparations (e.g. see Shimada *et al.*, 1986 and Sanchez-Ferrer *et al.*, 1988), wherein the effects have been found to vary between tissues and among species (Bhagavan *et al.*, 1993). In brief, VO_4 (1 mM) has been observed to cause a contractile response of variable magnitude in both vascular and non-vascular smooth muscle preparations *via* a mechanism that would appear not to involve the inhibition of Na^+/K^+-ATPase (summarized by Ozaki & Urakawa, 1980, Shimada *et al.*, 1986 and Sanchez-Ferrer *et al.*, 1988). Although it has been suggested that the contractile activity of VO_4 may be via inhibition of sarcolemmal Ca^{2+}-ATPase, causing elevation of intracellular calcium (Sanchez-Ferrer *et al.*, 1988), the ability of VO_4 to potentiate tyrosine kinase activity by inhibiting tyrosine phosphatase has not yet been explored in depth to account for its contractile effects.

Since we had already used tyrosine kinase inhibitors to evaluate a potential role for a tyrosine kinase pathway in the contractile actions of EGF-Uro and angiotensin II in gastric and vascular smooth muscle preparations (Yang *et al.*, 1992; 1993; Saifeddine *et al.*, 1992), we wished to use similar bioassay preparations to study the actions of the tyrosine phosphatase inhibitor, PV. The present study describes the characteristics of the contractile actions of VO_4 and PV in rat gastric longitudinal muscle and aortic ring preparations. Our data illustrate the inhibition of the contractile actions of PV by tyrosine kinase inhibitors (genistein and tyrphostin) and the correlation of PV-induced contractions with increases in tissue phosphotyrosyl protein content that could be attenuated by the tyrosine kinase inhibitors. The results support the hypothesis that PV can cause its effects on smooth muscle tension via the inhibition of a tyrosine phosphatase pathway, so as to potentiate the action of constitutively active tissue tyrosine kinase(s).

Methods

Bioassay procedures

Protocols for the humane treatment of animals, according to the Declaration of Helsinki, and as approved by an institutional committee on animal care were followed in all experiments. After the animals had been killed by cervical dislocation, the thoracic aorta and stomach were removed from male Sprague Dawley albino rats (250–300 g). Tissues were trimmed and aortic rings (either intact, or rubbed free of endothelium) and gastric longitudinal muscle strips were prepared essentially as previously described (Hollenberg *et al.*, 1993). In brief, gastric longitudinal muscle strips (3 × 10 mm) were obtained after trimming the tissue free from all mucosa, by cutting exactly across the visible circular muscle striations, under a dissecting microscope. Both the longitudinal muscle strips and aortic rings (2 × 3 mm) were mounted in a 4 ml organ bath and equilibrated for at least 60 min at 37°C, in a Krebs-Henseleit buffer, pH 7.4 of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 2.5, KH_2PO_4 1.2 and glucose 10.0, gassed continuously with 95% O_2:5% CO_2. In some experiments, sodium was replaced in this buffer by equimolar concentrations of either lithium or choline. To examine responses in the absence of calcium, tissues were switched to a calcium-free buffer, containing 1 mM EGTA 10 min prior to the addition of agonist, after responding to an agonist the tissues were then washed and re-equilibrated in a calcium-containing buffer. Optimal resting tension of both preparations was about 1 mN. Changes in muscle tension were monitored isometrically with either Statham or Gould force-displacement transducers. The presence (or absence) of an intact endothelium in an aortic ring preparation was monitored by evaluating the relaxation response to 1 μM acetylcholine, in a preparation that was precontracted submaximally (EC_{75}) with 0.1 μM noradrenaline. Unless otherwise indicated, tissues were exposed to agonists at 25 min intervals, and were allowed to contract to a plateau tension over a 5 to 10 min period, at which time the tissue was washed three times and re-equilibrated in fresh buffer. The contractile responses of different preparations to VO_4 and PV were normalized by expressing the contractile force as a percentage (% KCl) of the contractile response to 50 mM KCl (3.6 ± 0.5 mN for gastric strips; 1.4 ± 0.1 mN for aortic rings: means ± s.e.mean for $n = 12$). When present, the tyrosine kinase inhibitors (genistein or tyrphostin) were added to the organ bath 15 to 20 min prior to the addition of a contractile agonist. Compounds, as stock solutions, were added to the organ bath (4 ml) and final concentrations were calculated accordingly.

Chemicals and other reagents

Acetylcholine, carbachol, noradrenaline, angiotensin II, sodium vanadate, nifedipine, diltiazam, verapamil, indomethacin, tetrodotoxin and catalase (from bovine liver, 2000 u mg^{-1}) were from Sigma (St. Louis, MO, U.S.A.); genistein was from ICN Biochemicals (Costa Mesa, CA, U.S.A.) and tyrphostin (compound serial No. 23 described by Gazit *et al.*, 1989; also designated AG18) was synthesized according to Gazit *et al.* (1989). Stock solutions of genistein and tyrphostin were prepared in dimethylsulphoxide; the solutions were diluted so that the final concentration of dimethylsulphoxide in the organ bath was ⩽ 0.1% (v/v). This concentration of solvent alone had no effect on the bioassay preparations.

PV was generated essentially as described previously (Kadota *et al.*, 1987) by mixing stock solutions of sodium orthovanadate with an equimolar or molar excess of H_2O_2. After a 15 min period, the reaction was terminated by the addition of catalase (final concentration, 400 u ml^{-1}; 0.2 mg ml^{-1}) to metabolize unreacted H_2O_2. The reaction product, known to contain a number of peroxovanadium coordination compounds was added to the organ bath at least 15 min after initiating the catalase reaction, and within 1 h of preparation, as suggested previously (Kadota *et al.*, 1987; Fantus *et al.*, 1989). The final molarity of PV in the organ bath was calculated on the basis of the original concentration of orthovanadate in the reaction mixture.

Western blot analysis

Gastric longitudinal muscle or aortic ring preparations were mounted in an organ bath for equilibration according to the bioassay procedures described above. Equilibrated tissues were then exposed to PV either with or without pretreatment of the tissues for 20 min with genistein or tyrphostin, as for a contractile bioassay. At a time (about 2–5 min) just prior to the plateau of contraction, tissue was removed from the organ bath, quick-frozen on a solid CO_2-cooled plexiglass plate, chopped with a scalpel and immediately solubilized in 120 μl of boiling sample buffer (Laemmli, 1970), and clarified by centrifugation (microfuge, 2 min at approximately 20,000 *g*) in preparation for polyacrylamide gel electrophoresis (60 × 80 × 1.5 mm, 8% gel) and transfer (1.5 h at

100 V at 4°C) to nitrocellulose (0.45 μM, Bio Rad, Richmond, CA, U.S.A.) for Western blot detection of protein, after blocking the membrane for 1 h at 24°C with 1% w/v gelatin. As an alternative extraction procedure, quick-frozen tissue was placed in 10% trichloroacetic acid, acetone-dried and then solubilized in sample buffer as above, in preparation for gel electrophoresis. Both extraction methods yielded equivalent results. The total protein content of tissue extracts was estimated using the Folin reagent for a 5 to 10 μl aliquot that was first precipitated in 100 μl of 10% w/v trichloroacetic acid and rinsed with 100% ethanol; bovine albumin served as a standard. Equal amounts of protein (50 to 200 μg) were loaded onto the gel for each tissue sample. Monoclonal antiphosphotyrosine antibody (our clone 6D9) was prepared according to Glenney *et al.* (1988), purified by ion exchange chromatography, and used at a concentration of approximately 0.1 μg ml^{-1}. Antibody reacting with immobilized proteins on the Western blot (2.5 h at room temperature) was detected by enhanced chemiluminescence (Amersham, Oakville, Ont. Canada) using horseradish peroxidase-coupled sheep antimouse immunoglobulin G at high dilution (1/50,000 of manufacturers stock reacted with the membrane for 1 h at room temperature). Control experiments demonstrated that the signal yielded by the monoclonal antibody could be neutralized by a phosphotyrosyl hapten (either 5 mM phosphotyrosine or 25 mM phenylphosphate).

Results

Characteristics of the contractile responses to VO_4 and PV

Both VO_4 and PV caused a rapidly developing sustained contraction in both the gastric and aortic tissue assays (Figure 1, a and e). Upon washing the preparations free from VO_4 and PV, tension returned to baseline. The contractile responses caused by either VO_4 or PV in the two tissues were not sensitive to tetrodotoxin (1 μM) and were not blocked by atropine (1 μM), yohimbine (1 μM) or phenoxybenzamine (1 μM) (not shown). Thus, nerve-released neurotransmitters did not appear to play a role. In the aortic preparation, comparable contractile responses to either VO_4 or PV were observed either in the presence or absence of a functional endothelium (not shown).

In some respects, the tissue responses to VO_4 and PV differed. For instance, in the gastric longitudinal muscle tissue, although separate exposures at a fixed concentration of

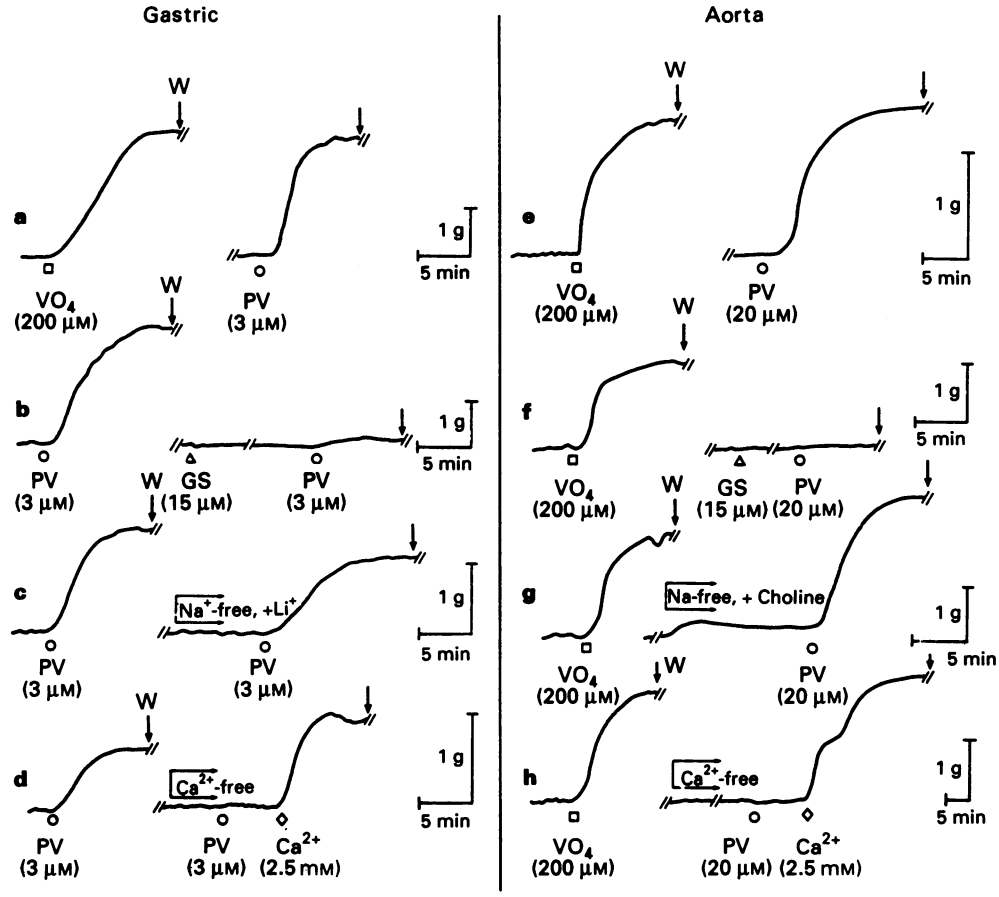

Figure 1 Responses of the gastric longitudinal muscle (LM) and aortic ring preparations to pervanadate (PV): comparison with vanadate (VO_4), effects of genistein and roles of extracellular sodium and calcium. Individual gastric (left: a to d) and aortic (right, e to h) tissue preparations were exposed first either to VO_4 (□, 200 μM) or PV (○, 3 μM) and tension was allowed to reach a plateau level (5–10 min) prior to washing (W, arrows) and re-equilibration. The same tissue was then either treated for 15 min (b and f) with genistein (GS: △, 15 μM) or was changed to a buffer in which sodium ion was replaced either with lithium (tracing c) or with choline (tracing g) or to a calcium-free buffer, containing 1 mM EGTA (tracings d and h). The response to PV (○, 3 μM) was again measured in the presence of 15 μM GS (b and f), in the absence of extracellular sodium (c and g) and in the absence of extracellular calcium (d and h). A contractile response was observed upon replenishing the calcium-free medium with 2.5 mM Ca^{2+} (d and h). Because of the desensitizing action of PV in the aortic tissue, control contractile responses were monitored first with VO_4 (□, 200 μM), prior to challenging each tissue only once with PV (○, 20 μM: tracings e to h). Each tracing (a to h), illustrating the response of an individual tissue preparation, is representative of four or more independently conducted experiments with tissues from four or more separate animals.

VO_4 (e.g. 200 μM) caused a reproducible contraction, without desensitization, repeated exposures of the tissue to a fixed concentration of PV (e.g. 3 μM) resulted in an increased contractile response, that stabilized after the third or fourth contraction. Thus, there appeared to be a 'kindling phenomenon' for PV but not for VO_4 in the gastric preparation. In the aortic tissue, as for the gastric longitudinal muscle tissue, repeated exposure to a fixed concentration of VO_4 caused a reproducible contractile response. However, in the aortic preparation, in contrast to the gastric tissue, concentrations of PV higher than 15 μM caused prolonged and generalized desensitization, in that upon washing the aortic tissue free from PV after the first robust contraction, the tissue became unresponsive for more than 2 h either to a second exposure to PV or to other contractile agonists such as noradrenaline (0.1 μM) or KCl (50 mM). Tissue responsiveness began to return after 3.5 h. These differences in the responses of the two tissues to VO_4 and PV were taken into account in the ensuing studies.

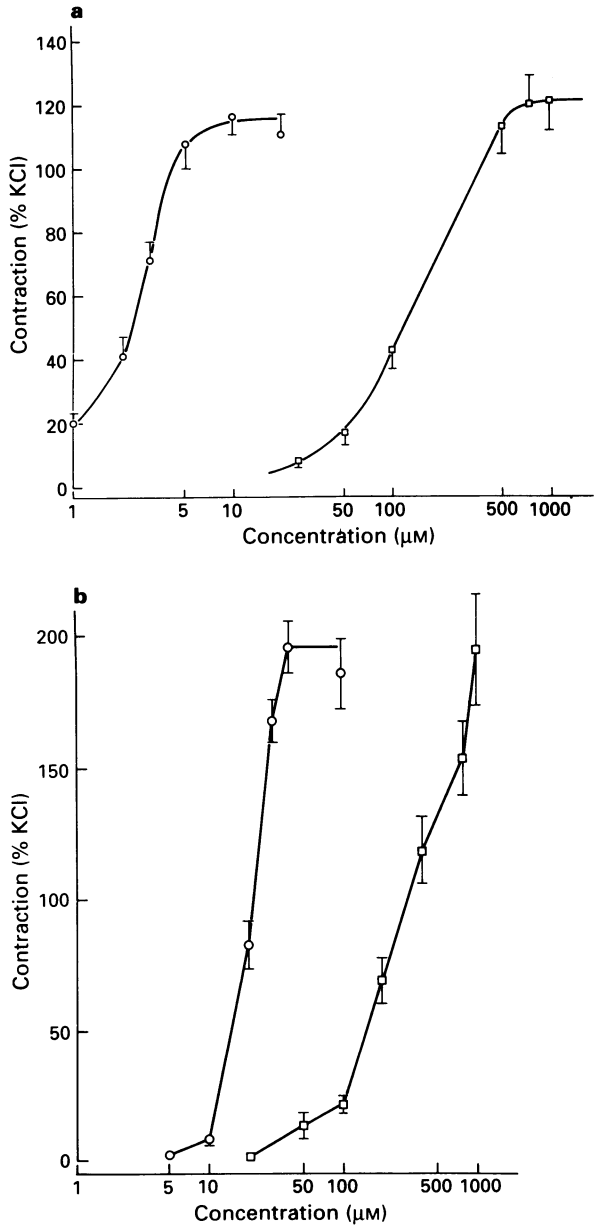

Figure 2 Concentration-effect curves for vanadate (VO_4) and pervanadate (PV). The contractile effects of increasing concentrations of either VO_4 (□) or PV (○) were measured in gastric (a) and aortic (b) preparations as described in the text. Responses in individual tissues are expressed as a percentage (% KCl) of a control response to 50 mM KCl. Values represent the means ± s.e.mean for measurements on 8 to 10 individual tissue preparations.

Concentration-effect curves

Gastric tissue In view of the characteristics of the responses to VO_4, it was possible, with the gastric preparation, to make sequential measurements of the contractile responses to increasing concentrations of VO_4 in individual matched tissue preparations (Figure 2a). The procedure for PV differed however, because of the 'kindling phenomenon' described above. Thus, for the PV concentration-effect curves, the gastric longitudinal muscle tissues were first primed several times with a low concentration of PV (3 μM) until the response had stabilized. At this point, the responsiveness to repeated exposures of either lower or higher concentrations of PV were also observed to be stable and reproducible; the concentration-response measurements for increasing concentrations of PV (Figure 2a) were then done sequentially in the primed tissue preparations.

Aortic tissue In the aortic preparation, the concentration-effect curves for VO_4 were obtained essentially as for the gastric longitudinal muscle preparation, using sequential measurements in individual tissues (Figure 2b). However, for evaluating PV action in the aortic preparation (Figure 2b), each tissue, after responding to a test concentration of KCl (50 mM) and noradrenaline (0.1 μM) (to provide an index of receptor-mediated tissue responsiveness), was subsequently exposed only once to PV. Figure 2 shows the contractile responses of the gastric and aortic preparations to VO_4 and PV expressed as a percentage of the contractile response elicited by 50 mM KCl; equivalent concentration-effect curves (not shown) were obtained when the contractile responses were expressed as a percentage of the responses to noradrenaline (aortic preparation only, 0.1 μM) or angiotensin II (gastric preparation only, 0.1 μM). The concentration-effect curves (Figure 2) showed that PV was approximately one to two orders of magnitude more potent than VO_4 in both tissues. For that reason, the subsequent experiments focused primarily on the contractile actions of PV alone.

Requirements for extracellular sodium and calcium

The contractile response of either the gastric or aortic tissue to PV was observed in the absence of extracellular sodium (Figure 1c and g). Nonetheless, in both the gastric longitudinal muscle and aortic preparations, the contractile

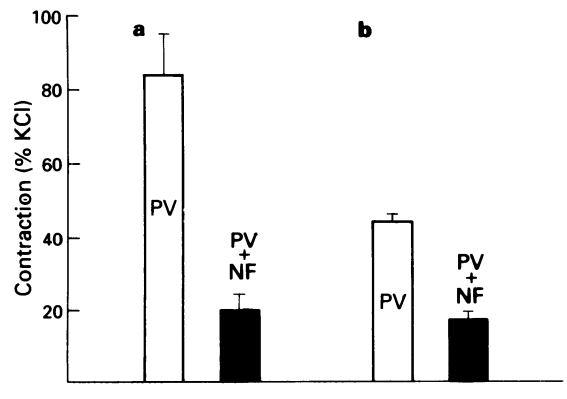

Figure 3 Effect of nifedipine (NF) on the contractile actions of pervanadate (PV). The contractile responses of aortic (a) and gastric (b) tissue preparations to PV were measured either in the absence (open columns) or presence (solid columns) of 1 μM nifedipine. The contractile responses to PV are expressed in each tissue as a percentage (% KCl) of the control contraction elicited by 50 mM KCl. Each histogram shows values for the means ± s.e.mean of measurements on four individual tissue preparations.

actions of PV were dependent on extracellular calcium (Figure 1d and h); a response to PV was not observed upon removal of calcium from the medium, but ensued upon restoring extracellular calcium. In keeping with this result, the calcium channel blocker, nifedipine, markedly attenuated the contractile action of PV in both preparations (Figure 3). Diltiazam and verapamil were much less effective than nifedipine in this regard (not shown).

Parallels between the actions of PV and EGF-Uro in the gastric longitudinal muscle preparation

In view of the ability of PV to mimic the action of a tyrosine kinase-mediated agonist such as insulin, we wondered if the characteristics of the contractile action of PV paralleled those of EGF-Uro in the gastric longitudinal muscle preparation, since EGF-Uro, like insulin, acts via a tyrosine kinase receptor. Because in our hands EGF-Uro did not cause contractions of the aortic tissue, we were not able to assess the parallels between the actions of PV and EGF-Uro in this preparation. As illustrated in Figure 4, there was indeed a parallel between EGF-Uro and PV action in the gastric tissue in terms of (1) the inhibition of the responses by the tyrosine kinase inhibitor, genistein, (2) the sensitivity of the response to indomethacin and (3) a requirement for extracellular calcium. In contrast, the contractile actions of carbachol (1 μM) in the longitudinal muscle preparation were not affected by either genistein or indomethacin and a contractile response to carbachol could be observed in the absence of extracellular calcium (Yang *et al.*, 1992 and data not shown).

Effects of tyrosine kinase inhibitors on contraction and on phosphotyrosyl protein content

We were interested to explore the concentration-range over which the tyrosine kinase inhibitors would affect the contractile actions of PV. As illustrated in Figure 1b and f, and by the concentration-inhibition curves in Figure 5, genistein at concentrations of 15 μM or below was able to attenuate selectively the effects of PV in both the gastric longitudinal muscle and aortic preparations, without causing a comparable inhibition of contractions elicited by KCl. The inhibitory effects were completely reversed upon washing the preparations free from genistein (not shown). The aortic preparation was particularly sensitive to the inhibitory actions of genistein. Tyrphostin was also able to block PV-mediated contractions selectively in the gastric longitudinal muscle preparation (Figure 5) but was unable, at comparable concentrations, to do so in the aortic preparation (not shown).

In view of the known ability of PV to inhibit protein tyrosine phosphatase, thereby potentiating the actions of tissue tyrosine kinases, we analysed, using a Western blot procedure, employing a monoclonal antiphosphotyrosine antibody, the phosphotyrosyl protein content of the gastric longitudinal muscle and aortic tissues after exposure to PV either in the absence or presence of either genistein (gastric and aortic preparations) or tyrphostin (gastric preparation only). As shown in Figure 6, PV, at the same time as it caused a contractile response, caused an increase in the Western blot reactivity of a number of constituents in both

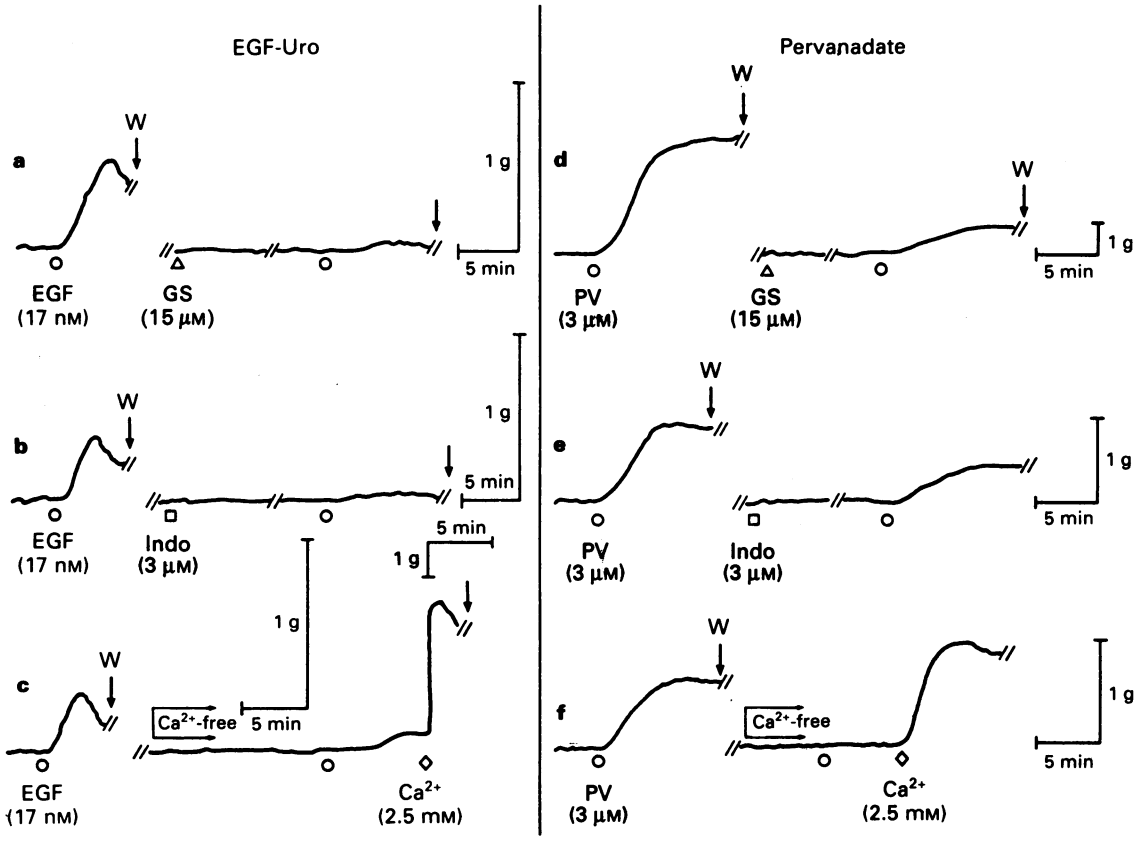

Figure 4 Comparison of the contractile actions of epidermal growth factor-urogastrone (EGF-Uro) and pervanadate (PV) in the gastric longitudinal muscle preparation: effects of genistein (tracings a and d) indomethacin (tracings b and e) and the absence of extracellular calcium (tracing c and f). Tracings for individual tissue preparations are shown, illustrating the responses to EGF-Uro (EGF, 17 nM: a,b,c) or PV (3 μM: d,e,f) either in the absence (before tissue wash, arrow, W) or presence of genistein (tracings a and d, GS: Δ, 15 μM) and indomethacin (tracings b and e, Indo: □, 3 μM). Tissues were treated with either genistein or indomethacin 20 min prior to the addition of EGF-Uro or PV to the organ bath. The effect of switching tissues to a calcium-free buffer containing 1 mM EGTA is also shown (tracings c and f) followed by replenishing the calcium (◇, 2.5 mM) in the continued presence of either EGF-Uro (tracing c) or PV (tracing f). Individual tracings for a single tissue are representative of three or more independently conducted experiments with tissues obtained from different animals.

the gastric and aortic tissues (Figure 6, lanes 1B and 2B); increases were observed prior to the development of maximum tension and were attenuated when the tissues were exposed to PV in the presence of genistein or tyrphostin (Figure 6, lanes 1C and 2C respectively). Genistein (not shown) was also able to reduce but not abolish the PV-mediated increases in gastric longitudinal muscle tissue phosphotyrosyl protein. The PV-mediated increases in tissue phosphotyrosyl protein content were also observed in the absence of extracellular calcium (not shown) under which conditions neither tissue exhibited a contractile response.

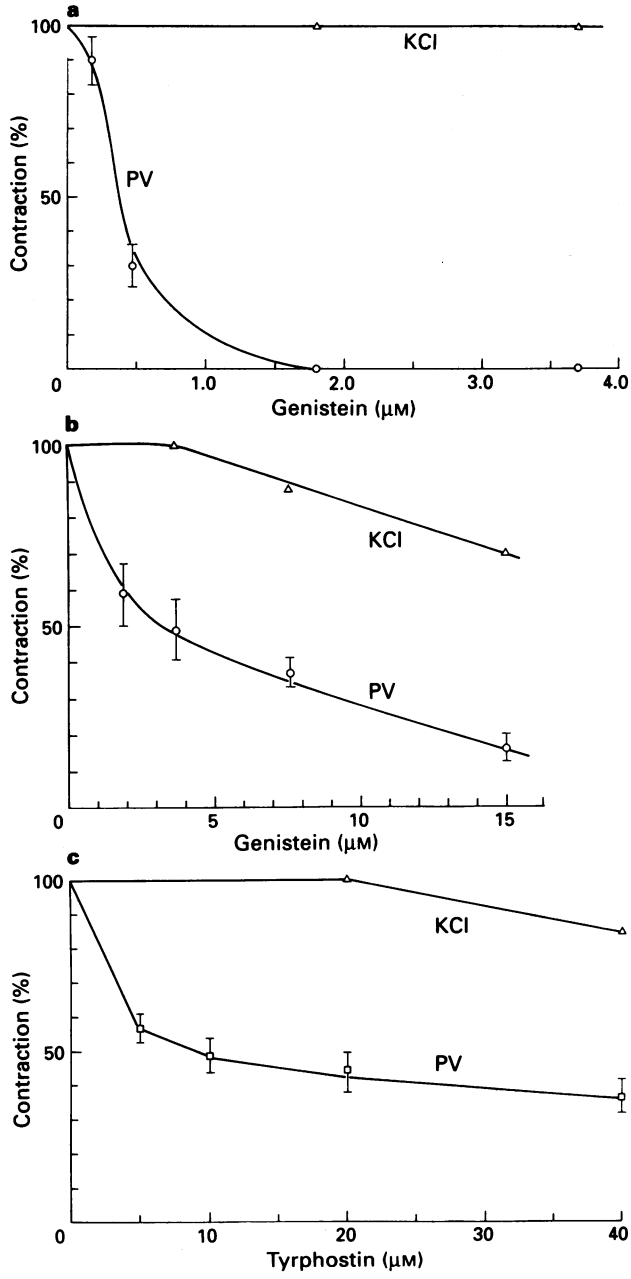

Figure 5 Concentration-effect curves for the inhibition of pervanadate (PV)-mediated contractions by tyrosine kinase inhibitors. The contractile responses to PV (20 μM for aortic tissue, a; 3 μM for gastric longitudinal muscle tissue, b and c) and to 50 mM KCl were measured in individual tissue preparations either in the absence or presence of increasing concentrations of either genistein (a and b) or tyrphostin (c) panel. Tissues were pretreated with genistein or tyrphostin 20 min prior to the addition of PV to the organ bath. The contractile responses of tyrosine kinase inhibitor-treated preparations were then expressed as a percentage (%) of the contraction observed in the absence of inhibitor. (a) Aortic tissue treated with genistein; (b) and (c): gastric tissue treated with either genistein (b) or tyrphostin (c). Values represent the means ± s.e.mean for experiments done with four or more independent tissue preparations.

Potentiation of angiotensin II action

Since our previous work had suggested a role for a tyrosine kinase pathway in the contractile effects of angiotensin (Saifeddine *et al.*, 1992; Yang *et al.*, 1993), we hypothesized that PV, by attenuating tyrosine phosphatase activity, might potentiate the action of angiotensin II. As illustrated in Figure 7, this proved to be the case for both the gastric longitudinal muscle and aortic ring assays, a concentration of PV, which by itself did not cause an appreciable contractile response, shifted the angiotensin II concentration-response curves for both preparations to the left, without affecting the maximum response.

Discussion

The main finding of our study was that PV, which has been found in other systems to be much more potent than VO_4 as an insulin-mimetic inhibitor of phosphotyrosyl phosphatase activity (Fantus *et al.*, 1989; Heffetz *et al.*, 1990; Bourgoin & Grinstein, 1992; O'Shea *et al.*, 1992), was also one to two orders of magnitude more potent than VO_4 in causing contractile responses in gastric and aortic smooth muscle preparations. The contractile responses to PV could not be attributed to the release of neurotransmitter or to the action,

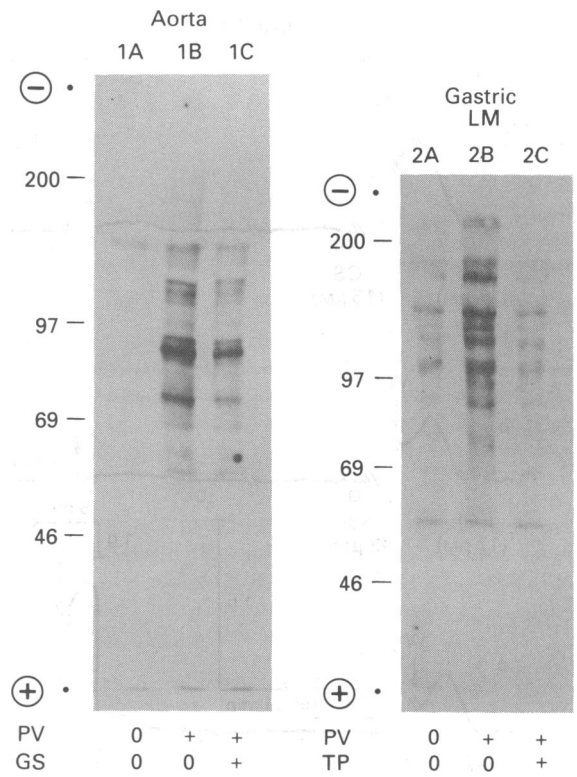

Figure 6 Western blot analysis of phosphotyrosyl proteins in pervanadate (PV)-treated tissues. Aortic (left, lanes 1A, 1B, 1C) and gastric (right, lanes 2A, 2B, 2C) tissues were recovered from the organ bath, as described in Methods, and processed for Western blot analysis. Tissues were either untreated (1A, 2A) or were exposed to PV either in the absence (1B, 2B) or presence of genistein (15 μM, lane 1C) or tyrphostin 80 μM, lane 2C. Molecular weight markers are shown on the left of each blot; the origin (⊕) and buffer front (⊖) are also indicated. The Western blot reaction shown for the monoclonal antiphosphotyrosine antibody was eliminated in the presence of 25 mM phenyl phosphate (not shown).

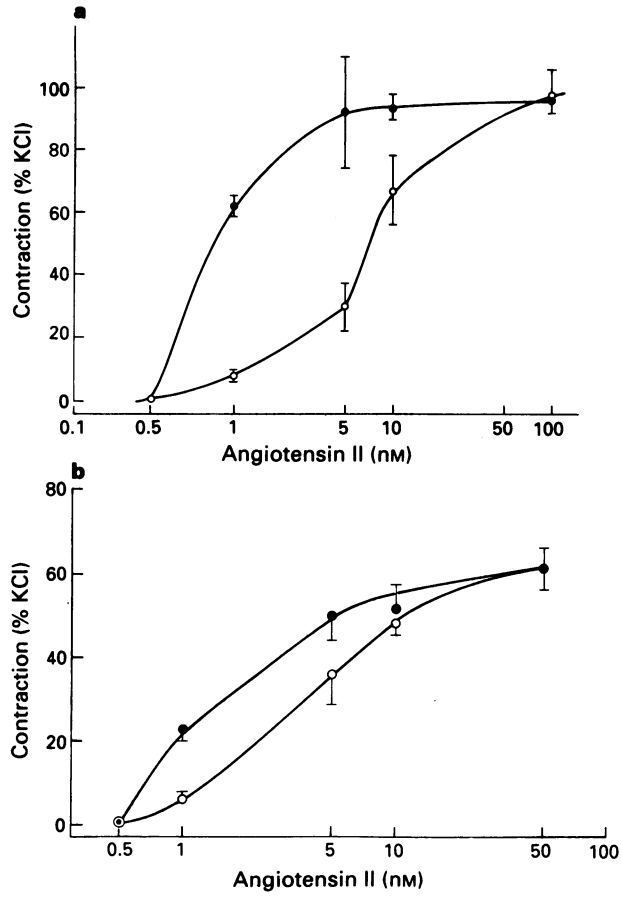

Figure 7 Potentiation of angiotensin II action by pervanadate (PV). The concentration-effect curves for the contractile action of angiotensin II in the gastric (b) and aortic (a) preparations were measured either in untreated tissues (○, control) or tissues that were pretreated with PV (●, 0.5 μM for gastric tissue; 10 μM for aorta tissue) prior to exposing the tissue to increasing concentrations of angiotensin. Values ± s.e.mean representing measurements made on 4 to 6 individual tissue preparations, are expressed as a percentage (% KCl) of the control contractile response in each tissue elicited by 50 mM KCl.

in the aortic preparation, of endothelial cell-released factors. Further, in accord with previous data ruling out a role for Na^+/K^+-ATPase inhibition in the contractile action of VO_4 (summarized by Fox *et al.*, 1983; Shimada *et al.*, 1986 and by Sanchez-Ferrer *et al.*, 1988), our observations that PV could cause its contractile effects in both the aortic and gastric preparations in the absence of extracellular sodium would argue against roles for Na^+/K^+-ATPase inhibition and Na^+/Ca^{2+} exchange in the PV-mediated contractile response. Additionally, there was a requirement for extracellular calcium. This calcium requirement for PV action is in agreement with other work documenting the importance of extracellular calcium for VO_4-induced contractions in rabbit vascular (Fox *et al.*, 1983) and gastric (Candura *et al.*, 1994) smooth muscle preparations. The dependence of the contractile effects on extracellular calcium would argue against the likelihood that PV was acting by inhibiting intracellular Ca^{2+}-ATPase, so as to provide an intracellular source of calcium to regulate the contractile apparatus. As an alternative, in view of the ability of the tyrosine kinase inhibitors to block the contractile action of PV, we suggest that it may be the inhibition of tyrosine phosphatase activity and thus, the potentiation of constitutive tyrosine kinase activity, that may best account for the contractile actions of PV.

One of the hallmarks of the pharmacological actions of VO_4 and PV is the simulation of the tyrosine kinase-mediated effects of insulin on its target tissues (Dubyak & Kleinzeller, 1980; Schechter & Karlish, 1980; Kadota *et al.*, 1987; Fantus *et al.*, 1989), in concert with increased protein tyrosine phosphorylation (Tamura *et al.*, 1984; Heffetz *et al.*, 1990). In a similar vein, our study showed that PV, in parallel with causing tyrosine kinase inhibitor-sensitive increases in tissue phosphotyrosyl protein content, mimicked the contractile action of EGF-Uro in the gastric preparation in terms of a sensitivity towards genistein and indomethacin and a requirement for extracellular calcium. These contractile response characteristics have been found to be typical for the tyrosine kinase-mediated contractile action of EGF-Uro but not for other agonists, such as carbachol, in a number of smooth muscle assay systems including gastric longitudinal muscle (Hollenberg, 1994). Thus, taken together, our data are entirely in keeping with the suggestion above, that the potentiation of a tyrosine kinase-mediated signal transduction pathway can account for the contractile actions of PV in the tissues we have examined.

Our work entirely supports and enlarges upon data that appeared upon completion of our study, demonstrating that VO_4-induced contractions in guinea-pig taenia coli preparations are associated with enhanced protein tyrosine phosphorylation (Di Salvo *et al.*, 1993a). Nonetheless, in our own work, the 1-D gel analysis of the phosphotyrosyl protein profiles of the gastric and aortic tissues exposed to PV in the absence and presence of the tyrosine kinase inhibitors did not single out any individual constituents, for which increased phosphorylation could be correlated directly with a contractile response. A more refined 2-D gel analysis of the phosphoproteins will be required to explore this potential correlation.

As yet, we have no explanation for the qualitative differences between the actions of PV and VO_4 on the gastric and aortic preparations (1) in terms of tissue desensitization (aorta) and (2) in terms of the 'kindling' phenomenon observed in the gastric preparation. Possibly these differences may relate to an increased potency of PV, compared with VO_4, for inhibiting cellular tyrosine phosphatase activity (Fantus *et al.*, 1989; Heffetz *et al.*, 1990; Faure *et al.*, 1992). Further, the differential effects of tyrphostin and the differences in the sensitivities of the two tissues to genistein may point to the involvement of distinct tyrosine kinases in the contractile responses of the two tissues, so as to lead to differences that would be observed upon blocking tyrosine phosphatase action. Alternatively, the differences may reflect a greater ability of PV to affect a variety of other enzyme systems (Bhagavan *et al.*, 1983; Erdmann *et al.*, 1984; Nechay *et al.*, 1986), in addition to the tyrosine phosphatase(s) we believe most likely to be responsible for initiating the rapid contractile response. Thus, although VO_4 and PV are often used interchangeably as probes for assessing the functional significance of tyrosine phosphatase in biological test systems, the two compounds may have distinct and complex actions on other enzyme pathways that may affect tissue responsiveness.

We believe the dependence of the contractile action of PV on extracellular calcium and the ability of PV to potentiate the contractile action of angiotensin II may relate to a role for tyrosine kinase activity in agonist-mediated calcium entry, as suggested by the work of Lee and co-workers (1993). This possibility is also in keeping with the ability of tyrosine kinase inhibitors to modulate voltage-sensitive calcium channels in isolated vascular smooth muscle cells (Wijetunge *et al.*, 1992) and with the ability of angiotensin II to stimulate protein tyrosine phosphorylation in smooth muscle tissue (Tsuda *et al.*, 1991; Huckle *et al.*, 1992; Molloy *et al.*, 1993; Yang *et al.*, 1993). Thus, the putative targets for tyrosine phosphorylation that could regulate tissue contractility might be components associated with the voltage-dependent calcium channel in the plasma membrane and would appear not to involve a downstream regulation of the contractile apparatus *per se*. This suggestion is in accord with our observation that PV-triggered cellular tyrosine phosphorylation, occurring in the absence of extracellular calcium, did

not lead to a contractile response. Thus in summary, our data support the hypothesis that PV causes its contractile effects via the inhibition of a tyrosine phosphatase pathway, thereby amplifying constitutive tyrosine kinase activity, so as to facilitate the calcium entry through voltage-sensitive calcium channels. We believe that such a role for tyrosine phosphorylation/dephosphorylation in the control of smooth muscle tension may be relevant to the action of a variety of G-protein-coupled and growth factor receptor-coupled contractile agonists.

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