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Effect of inhibition of tyrosine phosphatases on voltage-operated calcium channel currents in rabbit isolated ear artery cells

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1 The effect of increasing cellular tyrosine phosphorylation by inhibiting endogenous tyrosine phosphatases was examined on voltage-operated calcium channel currents in vascular smooth muscle cells.

2 In single ear artery smooth muscle cells of the rabbit, studied by the whole cell voltage clamp technique, intracellular application of the tyrosine phosphatase inhibitors, sodium orthovanadate (100 μM) and peroxyvanadate (100 μM orthovanadate + 1 mM H_2O_2) increased voltage-operated calcium channel currents by 56% and 83%, respectively.

3 Bath application of two other membrane permeant tyrosine phosphatase inhibitors, phenylarsine oxide (100 μM) and dephostatin (50 μM) also increased voltage-operated calcium channel currents by 48% and 52%, respectively.

4 The selective tyrosine kinase inhibitor, tyrphostin-23 (100 μM) reduced calcium channel currents by 41%. Pre-incubation with tyrphostin-23 abolished the effects of peroxyvanadate, phenylarsine oxide and dephostatin on calcium channels.

5 Western blot analysis of rabbit ear artery cell lysates showed increased tyrosine phosphorylation of several endogenous proteins following treatment with peroxyvanadate.

6 These results indicate that a number of structurally dissimilar inhibitors of tyrosine phosphatases increase voltage-operated calcium channel currents in arterial smooth muscle cells presumably due to increased tyrosine phosphorylation.

Keywords: Voltage-operated calcium channels; tyrosine phosphorylation; tyrosine phosphatases

Introduction

Voltage-operated calcium channels (VOC) are the major route of calcium entry into vascular smooth muscle cells and are regulated by a variety of cellular processes including protein phosphorylation (reviewed in Hughes, 1995). The role of serine and threonine kinases such as protein kinase C (Loirand *et al*., 1990) and adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent kinases (Fukumitsu *et al*., 1990; Tewari & Simrad, 1994) in regulating VOC is well established. Recent studies have indicated a role for protein tyrosine kinases as modulators of calcium channels in smooth muscle cells (Wijetunge *et al*., 1992) and other cell types (Slomiany *et al*., 1992; Cataldi *et al*., 1996). Protein tyrosine kinases (PTKs) are enzymes responsible for a number of signalling processes in the cell particularly those associated with cell growth (Schlessinger & Ullrich, 1992), though more recently PTKs have also been implicated in processes unrelated to growth such as smooth muscle cell contraction (reviewed in Hollenberg, 1994). There are two major types of PTKs: receptor-linked tyrosine kinases such as the platelet-derived growth factor (PDGF) receptor-linked and epidermal growth factor (EGF) receptor-linked tyrosine kinases, and the non-receptor tyrosine kinases such as the Src family of tyrosine kinases and the products of Abl and Fes (Toyoshima *et al*., 1992). Evidence exists that both receptor and non-receptor tyrosine kinases may modulate calcium channels in vascular smooth muscle cells. We have previously shown that extracellular application of PDGF (Wijetunge & Hughes, 1995a) and intracellular application of the cytoplasmic non-receptor tyrosine kinase (human $pp60^{c\text{-}Src}$) (Wijetunge & Hughes, 1995b) increased calcium channel currents in arterial smooth muscle cells. We have also shown previously that selective PTK inhibitors such as tyrphostin-23, genistein and peptide-A, a 21 amino acid inhibitor of Src, reduce VOC currents in arterial cells, suggesting a role for an endogenous tyrosine kinase active under resting conditions in modulating VOC (Wijetunge *et al*., 1992). c-Src is a possible candidate for this role since it is found in large amounts in smooth muscle cells (Di Salvo *et al*., 1989) and may maintain a level of activity under unstimulated conditions, possibly as a result of cell-matrix interactions (Malik & Parsons, 1996). We have provided further support for this proposal by showing recently that a 11 amino acid peptide (EPQY(PO_3H_2)EEI-PIYL) containing the sequence (pY)EEI which selectively activates endogenous c-Src (Liu *et al*., 1993) increased calcium channel currents in vascular smooth muscle cells (Wijetunge & Hughes, 1996). Interestingly recent studies have indicated that in addition to being activated by growth factors such as PDGF and EGF (Courtneidge, 1994) c-Src may also be stimulated by contractile agents linked to heterotrimeric G-protein coupled vasoconstrictors such as angiotensin II (Ishida *et al*., 1995) and endothelin (Simonson & Herman, 1993) and thus could mediate the actions of a range of smooth muscle stimulants.

In contrast with the increasing information regarding tyrosine kinases in smooth muscle, relatively little is known regarding the role of tyrosine phosphatases in vascular smooth muscle. Endogenous tyrosine phosphatases (PTP) play an important role in signal transduction mechanisms involving tyrosine phosphorylation by counteracting the action of protein tyrosine kinases. PTPs are a diverse family of enzymes widely distributed in a variety of cell types (Lau *et al*., 1989). Inhibition of the action of endogenous PTPs prevent dephosphorylation of tyrosine residues so that PTK activity in the cell would be unopposed, leading to an increase in overall tyrosine phosphorylation of cellular proteins. In addition, as

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several PTKs, including c-Src, are themselves activated by phosphorylaton of critical tyrosine residues (Courtneidge, 1994), inhibition of PTP activity may also directly increase activity of these endogenous tyrosine kinases. Previous studies in single smooth muscle cells of the rabbit colonic muscularis mucosae (Hatakeyama *et al*., 1996), and pituitary GH3 cells (Cataldi *et al*., 1996) have showed that vanadate activated dihydropyridine-sensitive calcium channel currents. Therefore the objective of this study was to investigate the effect of increased tyrosine phosphorylation on VOC by using a range of different PTP inhibitors, sodium orthovanadate, peroxyvanadate, phenylarsine oxide and dephostatin.

Methods

Single smooth muscle cells were freshly dispersed from rabbit ear arteries by an enzymatic method previously described (Benham & Bolton, 1986). Short (2–3 mm) segments of artery were incubated for 50 min in a modified physiological salt solution (PSS) containing (mM): NaCl 130, KCl 6, $CaCl_2$ 0.01, $MgCl_2$ 1.2, glucose 14 and HEPES 10.7 buffered to pH 7.2 with NaOH, 2 mg ml^{-1} bovine serum albumin (BSA), 1 mg ml^{-1} collagenase (130 u mg^{-1}), 0.5 mg ml^{-1} papain (15 u mg^{-1}) and 5 mM dithiothreitol. Cells were dispersed by trituration of the tissue through a blunt nosed Pasteur pipette and resuspended after centrifugation in normal PSS containing 1.7 mM $CaCl_2$. Cells were stored on cover slips at 4°C and used within 4–6 h. Internal pipette solution contained (mM): NaCl 126, $MgCl_2$ 1.2, EGTA 2, MgATP 2, TEA 10, and HEPES 11 buffered to pH 7.2 with NaOH. Patch pipettes were fabricated from borosilicate glass and had resistances of 3–5 MΩ.

Calcium channel currents were measured by the whole cell configuration of the voltage clamp technique (Hamill *et al*., 1981) with a List EPC-7 amplifier, Labmaster A/D interface board with commercially available software (Pclamp 5.5, Axon Instruments CA, USA) on an IBM compatible PC. The experiments measuring voltage-operated calcium channel currents were carried out in a 'high barium' solution ($BaCl_2$ 110 mM, HEPES 10 mM; buffered to pH 7.2 with TEA-OH) to increase the size of the inward current and to minimize calcium-dependent inactivation of currents (Aaronson *et al*., 1988). Data were recorded on-line, or on digital audio tape with a DAT recorder (Biologic, France), and analysed off-line after analogue-to-digital conversion using PClamp 5.5 software on a PC. The currents were digitally filtered at 2 kHz and leak currents were subtracted digitally, with average values of steady leakage currents elicited by a 10 mV hyperpolarizing pulse (Aaronson *et al*., 1988). All recordings were made at room temperature (22–25°C).

Concentrations in excess of the EC_{50} values were used for all drugs. Stock solutions of phenylarsine oxide (PAO; 1 mM) and dephostatin (50 mM) were made up in distilled water and dimethylsulphoxide (DMSO) respectively and stored at −20°C. Final concentrations of 100 μM PAO and 50 μM dephostatin were made up in the 'high barium' solution and applied to the cell by bath perfusion. A 50 mM stock solution of the tyrosine kinase inhibitor tyrphostin-23 was made up in DMSO and stored at −20°C. This was diluted in the 'high barium' solution to a final concentration of 100 μM before the experiments. This solution containing 100 μM tyrphostin-23 was used to pre-incubate the cells for at least 10 min before the addition of the PTP inhibitors. Final concentration of DMSO did not exceed 0.2% which was itself without effects on VOC. A 200 mM stock solution of sodium orthovanadate was made up in distilled water and was applied intracellularly by inclusion in the internal pipette solution at a final concentration of 100 μM. Peroxyvanadate was made up by mixing 100 μM sodium orthovanadate with 1 mM H_2O_2 as previously described (Kadota *et al*., 1987), and this was done immediately before it was applied to the cell by including it in the internal pipette solution. To minimize batch to batch variations, the effects of the phosphatase inhibitors in the absence and presence of tyrphostin-23 was examined in cells obtained from the same isolates.

Preparation of rabbit ear artery cell lysate

Rabbit cells were isolated exactly as described above. The cells in PSS were treated with peroxyvanadate for 5 min. After centrifugation at 1000 r.p.m. for 5 min the supernatant was discarded and the cell pellet was resuspended in 50 μl lysis buffer, containing (mM): Tris 50, NaCl 150, EGTA 1, NP-40 1% (v/v), sodium deoxycholate (19% w/v), aprotinin, leupeptin, pepstatin (all at 1 μg ml^{-1}) and PMSF (200 μM) and allowed to stand on ice for 5 min before homogenization with a glass-on-glass homogenizer. The lysed cell samples were centrifuged at 4°C at 15,000 r.p.m. for 15 min and the supernatant transferred to fresh cold eppendorfs. A protein assay was carried out with a BCA protein assay kit. The samples were diluted with lysis buffer in order to achieve the same concentration of protein in each sample before they were heated at 95°C for 5 min with 5× sodium dodecyl sulphate (SDS) sample buffer containing Tris-HCl (pH = 6.8) 0.3 mM, β-mercaptoethanol 25% v/v, SDS 10% v/v, glycerol 50% v/v bromophenol blue 0.01% w/w. The samples were stored at −20°C used for gel electrophoresis.

BCA protein assay

Determination of protein concentration in the samples were carried out with a BCA protein assay kit (Pierce, U.S.A.) based on the method described by Bradford (1976).

SDS-polyacrylamide gel electrophoresis

SDS–PAGE was carried out with a Bio Rad Protean II system. Equal amounts of protein samples (15 μg) and molecular weight markers were loaded into wells and the gel was run in SDS running buffer (Tris 25 mM, glycine 192 mM and SDS 1% w/v). The separated proteins were transferred onto supported nitrocellulose membrane with a Bio Rad transfer cell and transfer buffer (Tris 25 mM, glycine 192 mM and methanol 20% v/v) at 35 V overnight. After transfer the membrane was blocked for 1–3 h with 5% BSA in Tris buffered saline (TBS) containing 0.001% v/v Tween 20 (TTBS). The blot was then probed with a monoclonal antiphosphotyrosine (PY20) primary antibody at 1/1000 dilution for 1 h. This was followed by a thorough wash cycle (2 quick rinses followed by 3 × 5 min washes followed by a 15 min wash) with TTBS to remove unbound primary antibody. The primary antibody was detected by use of a horseradish peroxidase conjugated-secondary antibody (1/1000 dilution) after incubation for 1 h. Following a similar wash cycle as above the antigen-antibody complex was visualized by enhanced chemiluminescence.

Drugs and chemicals

Acrylamide, hybond C nitrocellulose membranes, enhanced chemiluminescence reagents (Amersham, U.K.). Bio Rad Protean II system for gel running and transfer cell (BioRad,

Hemel Hempstead U.K.), BCA protein assay kit (Pierce Warner, Chester, U.K.), collagenase (Worthington, Reading, U.K.), dephostatin (LC laboratories, U.K.), PY20 (Affinity, Exeter, U.K.), HRP-linked rabbit anti-mouse antibody (Dako Ltd, Cambridgeshire, U.K.), (−)202791 ((−)4amino-5-(4methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimadine) (Gift from Sandoz, Switzerland) and all other chemicals from Sigma (Dorset, U.K.).

Statistics and data analysis

Current-voltage (*I*-V) relationships were obtained by repeated, progressive depolarization to various test potentials for 200 ms from a holding potential of −60 mV. The effect of a drug on *I*-V relationship was examined after any response to the drug had stabilized (approximately 3–5 min after application). The peak inward current at each test potential was measured. To investigate the effect of peroxyvanadate on the voltage-dependence of activation and inactivation, activation and inactivation curves were calculated. The activation curve was derived from the current-voltage relationships. Conductance (g) was calculated from the equation: $g = I_{Ba}/(E_m\text{-}E_{rev})$, where I_{Ba} is the peak current elicited by depolarizing test pulses to various test potentials (E_m). E_{rev} is the reversal potential estimated from the current-voltage curves. The conductance data were fitted to a Boltzman function: $g = 1/[1+\exp\{(E_m\text{-}V_h)/k\}]$, where V_h is the potential required for half activation and k is the slope factor.

The steady-state inactivation curves were obtained from a double-pulse protocol. Following a 6 s conditioning pulse to various potential to induce steady-state inactivation, currents were evoked by depolarizing to +20 mV from a holding potential of −60 mV. The data were fitted to a Boltzman equation: $I = [I_{max}/1+\exp\{(E_m\text{-}V_h)/k\}]+I_{non}$, where I is the peak current at any potential, I_{max} is the maximum peak current evoked by such a step, V_h is the potential at which current is half inactivated, k is a slope factor and I_{non} is the non-inactivating fraction of current. Data were fitted by non-linear regression analysis by use of Microsoft Excel.

To compare the kinetics of activation and inactivation of currents in control cells and peroxyvanadate-stimulated cells, currents were evoked by a depolarizing pulse to +20 mV for 500 ms from a holding potential of −60 mV. Following subtraction of capacitance artifacts, the data were fitted to a double exponential function: $I(t) = a_1\exp^{-t/\tau_1} + a_2\exp^{-t/\tau_2} + a_3$ by using PClamp 5.5 software, where $I(t)$ is the amplitude of the current at time t, a_1, and a_2 and a_3 are the amplitudes of the activation, inactivation and non-inactivating components, respectively; τ_1 and τ_2 are the time constants of the activation and inactivation components.

Data are presented as means ± s.e.means of (n) observations. Comparisons of data were made by a Student's t test for single paired comparisons and $P<0.05$ was considered statistically significant.

Results

Previous studies (Benham *et al*., 1987; Aaronson *et al*., 1988) have indicated that the inward Ba^{2+} current in rabbit ear artery cells is predominantly due to influx through L-type channels. In keeping with these earlier studies inward Ba^{2+} current induced by depolarization from a holding potential of −60 mV was abolished by 5 μM (−)202791, a dihydropyridine L-type calcium channel antagonist (Hering *et al*., 1989) (Figure 1). In 9 cells examined, the average peak current at +20 mV, following application of (−)202791 was −2.7 ± 1.4 pA. Since T-type currents are known to be activated at membrane potentials 20–40 mV more negative than L-type channels, currents evoked from holding potential of −60 mV were compared to those evoked from −90 mV. No significant difference in the threshold of activation or the potential at which peak current was elicited (V_{peak}) were seen (Figure 1), suggesting negligible contribution from T-type channels to voltage-activated inward currents carried by Ba^{2+} seen in these studies.

In rabbit ear artery cells the inclusion of sodium orthovanadate (100 μM), in the pipette solution increased calcium channel currents elicited every 1 s by a 20 ms step to +10 mV from a holding potential of −60 mV, from 36.3 ± 5 pA at establishment of whole cell mode to a stable value of 56.8 ± 9 pA over a 3–5 min period. This effect corresponded to a 56 ± 16% increase in current ($n=4$, $P<0.05$, Figure 2). Similarly, inclusion of a related but more potent inhibitor of PTP, peroxyvanadate (PV) (100 μM sodium orthovanadate + 1 mM H_2O_2) in the pipette solution increased calcium channel currents evoked by a similar activation protocol over a similar time period, from 48 ± 3 pA to 86 ± 10 pA, which corresponds to a 83 ± 20% increase in current ($n=6$, $P<0.007$, Figure 3). Examination of currents under control conditions with Ba^{2+} as the charge carrier and NaCl containing intracellular pipette solution, showed that over a 3 min period there was no increase or 'run up' of current, and negligible rundown of current ($n=5$, Figure 3c).

Examination of the *I*-V relationship in cells dialysed with PV for in excess of 5 min indicated that PV increased the

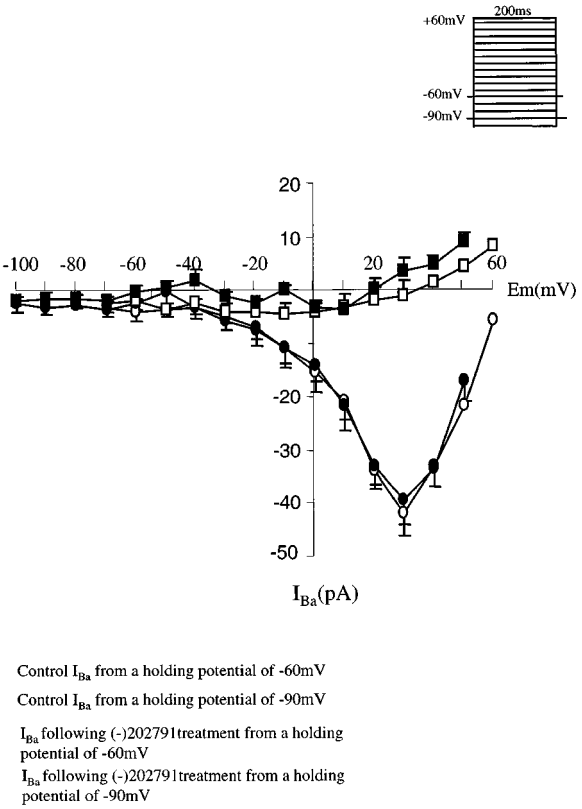

Figure 1 The current-voltage (*I*/V) relationships of calcium channel currents in a single cell. *I*/V relationships were derived from a holding potential (Hp) of −60 mV or from −90 mV by depolarizing to various test potentials for 200 ms in the presence or absence of a dihydropyridine (DHP) calcium channel antagonist (−)202791 5 μM. *I*/V data were measured at least 3–4 min after establishment of whole cell mode when the size of the currents had stabilized. The mean data from 9 cells is shown; vertical lines indicate s.e.mean.

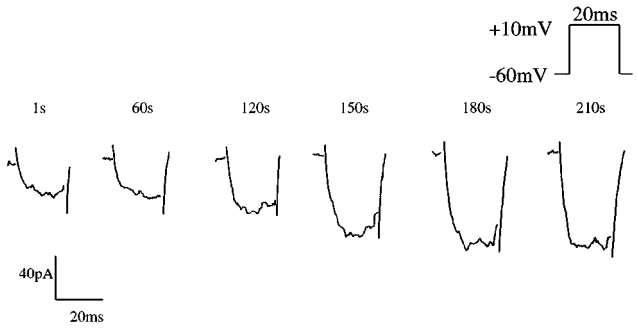

Figure 2 Effect of sodium orthovanadate (SV) (100 μM) on voltage-operated calcium channel currents in a single rabbit ear artery cell. SV was included in the intracellular pipette solution and allowed to dialyze into the cell following establishment of whole cell mode. Calcium channel currents were measured by use of the whole cell voltage clamp technique with 110 mM Ba^{2+} as the charge carrier. Currents were evoked every 1 s following the achievement of whole cell mode with a 20 ms depolarizing step to +10 mV from a holding potential of −60 mV. The figure shows current traces recorded from a representative individual cell at 1 s, 60 s, 120 s, 150 s, 180 s and 210 s following the establishment of whole-cell mode and traces are representative of 4 similar experiments.

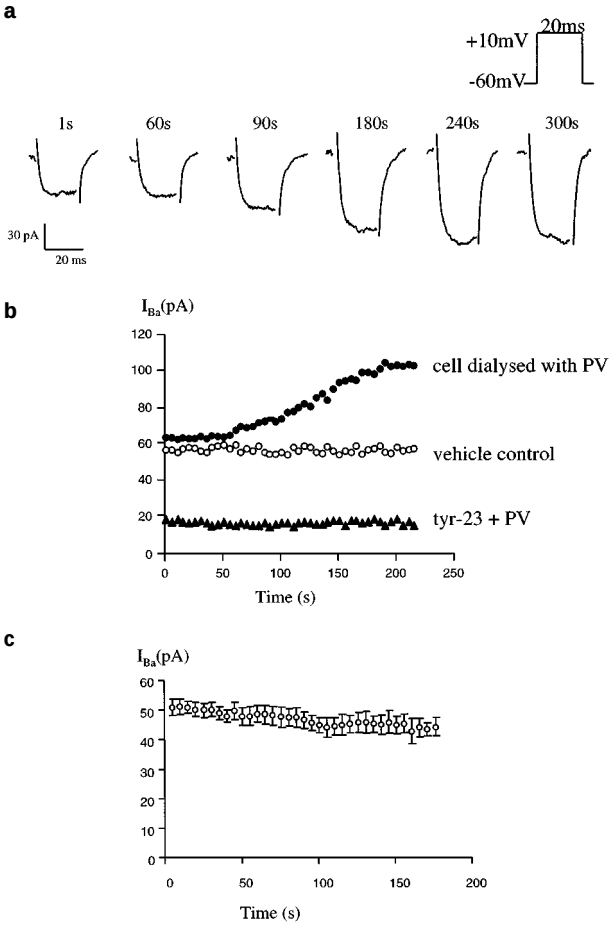

Figure 3 (a) Effect of peroxyvanadate (PV) on voltage-operated calcium channel currents in a single rabbit ear artery cell. Peroxyvanadate was included in the intracellular pipette solution and allowed to dialyze into the cell following establishment of whole cell mode. Calcium channel currents were measured by the whole cell voltage clamp technique with 110 mM Ba^{2+} as the charge carrier. Currents were evoked by a similar protocol as in Figure 1. The current traces were recorded from a representative individual cell (of 6 similar experiments) at 1 s, 60 s, 90 s, 180 s, 240 s and 300 s following the establishment of whole-cell mode. (b) Onset of effect of peroxyvanadate (PV, 100 μM sodium orthovanadate with 1 mM H_2O_2) on voltage-operated calcium channel currents. PV was contained in the intracellular pipette solution. The calcium channel currents were recorded from 3 individual cells following establishment of whole cell mode. Each dot in the figure represents the mean current over a 5 s period. Results are representative of 4–6 similar experiments. (c) Calcium channel currents recorded under control conditions. Intracellular pipette solution, containing (mM): NaCl 126, $MgCl_2$ 1.2, EGTA 2, MgATP 2, TEA 10 and HEPES 11, was allowed to dialyze into the cell after establishment of whole cell mode. Calcium channel currents were measured by the whole cell voltage clamp technique with 110 mM Ba^{2+} as the charge carrier. Currents were evoked by a similar protocol as in Figure 1. Average calcium channel currents are shown from 5 cells following establishment of whole cell mode recorded over 3 min; vertical lines indicate s.e.mean. Each dot in the figure represents the mean current over a 5 s period.

inward currents over a range of test potential (0 to +60 mV), without affecting the potential at which the peak of the *I*-V curve was evoked (Figure 4). Analysis of the activation curves showed no change in the potential for half-maximal activation by PV. Inspection of individual current traces showed that the kinetics of activation and inactivation of currents were also not affected by PV (for control cells, activation time constant (τ_1) = 1.45±0.1 and inactivation time constant (τ_2) = 86±2; for PV induced currents τ_1 = 1.6±0.3 and τ_2 = 93±3, n = 3 in both cases, Figure 4).

Analysis of the activation curve derived from the *I*-V relationship showed that neither the potential for half maximal activation (Vh_{act}) nor the slope were significantly shifted by PV (Figure 5a). Vh_{act} = 6.2±2 mV and Vh_{PV} = 6.0±2 mV. Slope = 11.4±1 (control) and 11.3±1(PV) (n = 4 in both cases). PV also did not affect the position or shape of the steady-state inactivation curve for calcium channel currents (Figure 5). The potential for half maximal inactivation, V_h was −19.7±1 mV in control and −17.3±3 mV following PV, and the slope = −10.5±1 mV^{-1} (control, n = 4) and −9.2±1 mV^{-1} (PV, n = 5).

To examine whether the effect of PV on calcium channels was due to increased tyrosine phosphorylation, we examined the effect of PV following pre-incubation with a selective tyrosine kinase inhibitor, tyrphostin-23 (100 μM). Tyrphostin-23 reduced currents evoked by a 20 ms step to +10 mV from a holding potential of −60 mV, from 40.8±2 pA to 23±3 pA which corresponds to 44±4% inhibition of currents (n = 4, P = 0.001) as we have previously shown (Wijetunge *et al.*, 1992). In cells pre-incubated for over 10 min with 100 μM tyrphostin-23, inclusion of PV in the pipette solution failed to increase calcium channel currents significantly (n = 4, Figures 3 and 8).

Addition of 100 μM phenylarsine oxide (PAO), another PTP inhibitor, to the bath solution increased calcium currents elicited every 1 s by a 20 ms step to +10 mV from a holding potential of −60 mV. The mean inward current increased from 36±4 pA to 55±7 pA corresponding to a 48±7% increase in current (n = 9, P < 0.0005, Figure 6). PAO increased currents over a range of test potentials (0 to +60 mV) but did not affect the voltage at which the peak of the *I*-V curve was evoked (Figure 6). Since PAO is thought to act by an oxidative mechanism which is inhibited by reducing agents, we examined the effect of a reducing agent dithiothrietol (DTT) on the effect of PAO. Dithiothreitol (DTT) 1 mM reversed the effect of PAO (n = 3, Figure 5a). DTT (1 mM) on its own did not have any significant effects on channel currents (control = 45.8±4 pA, DTT = 48.3±9 pA, n = 4, Figure 5b). Pre-incubation of cells with tyrphostin-23 (100 μM) abolished the effect of PAO (n = 5, Figure 8).

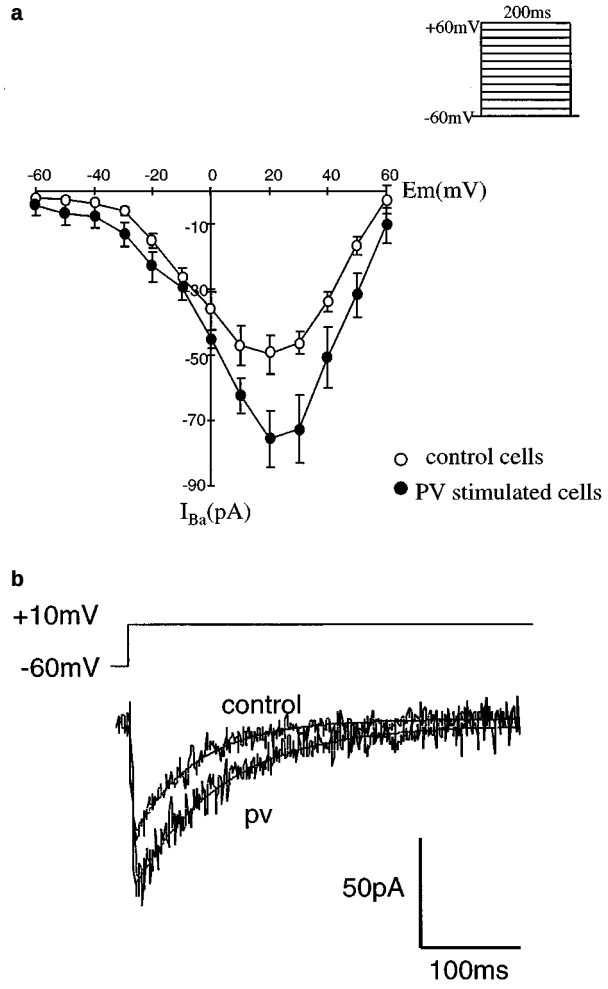

Figure 4 (a) Effect of peroxyvanadate on the mean current-voltage (I/V) relationship of calcium channel currents. I/V relationships were derived from a holding potential of −60 mV by depolarizing to various test potentials for 200 ms as shown in the protocol (insert). I/V data were measured at least 3–4 min after establishment of whole cell mode when the size of the currents had stabilized. Data are means of 4–6 observations; vertical lines indicate s.e.mean. (b) Effect of PV on kinetics of activation and inactivation of calcium channel currents in a single cell. Currents were evoked by a 500 ms depolarizing step to +10 mV from a holding potential of −60 mV. Data were fitted by a bi-exponential function including a component for non-inactivating current (see Methods). The non-linear regression line is shown in bold, c = control, pv = peroxyvanadate. For the examples shown $\tau_1 = 0.7$ ms, $\tau_2 = 61$ ms for control and $\tau_1 = 1.3$ ms, $\tau_2 = 79$ ms for peroxyvanadate ($r^2 > 0.9$ in both cases). The traces are representative of 3 similar experiments.

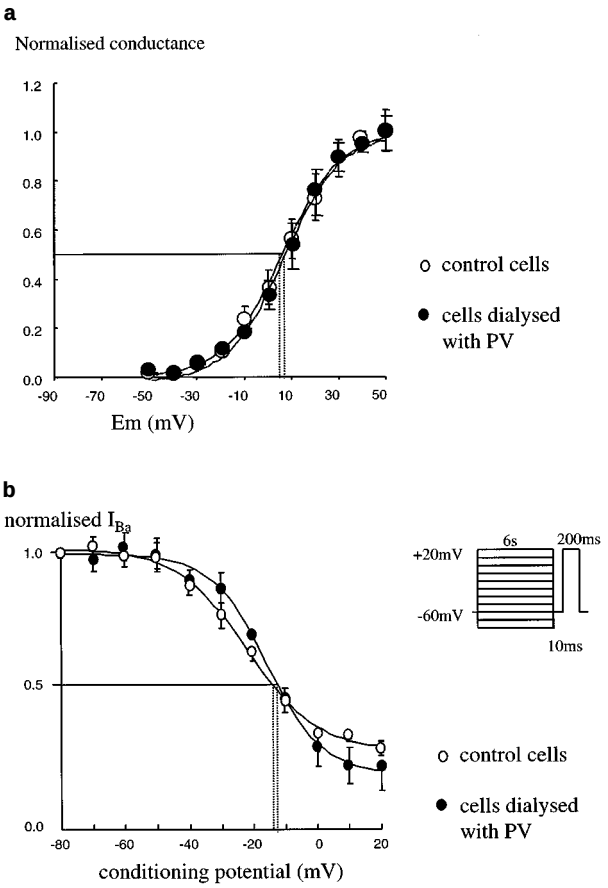

Figure 5 (a) Effect of peroxyvanadate on the activation curve for calcium channels showing normalized conductance as a function of test potential (Em). The activation curve was derived from the current-voltage relationships. Conductance(g) was calculated from the equation, $g = I_{Ba}/(E_m - E_{rev})$, where I_{Ba} is the peak current elicited by depolarizing test pulses to various test potentials (E_m). E_{rev} is the reversal potential estimated from the current-voltage curves. The conductance data were fitted to a Boltzman function as follows: $g = 1/[1 + \exp\{(E_m - V_h/k\}]$, where V_h is the potential required for half-activation and k is the slope factor. Data are means of 4 observations. (b) The effect of peroxyvanadate on the steady-state inactivation of calcium channel currents. Cells were held at various conditioning voltages for 6 s to achieve inactivation, then held for 10 ms at the holding potential of −60 mV before currents were evoked by a 200 ms depolarizing step to +20 mV, as shown in the voltage protocol I_{Ba} normalized with respect to I_{Ba} evoked following a −80 mV conditioning potential is plotted against the conditioning potential. Inactivation data were measured at least 5–6 min after establishment of whole cell mode when the magnitude of I_{Ba} had stabilized. Data were fitted to a Boltzman equation as described in Methods. Data are means of 4–5 observations: In (a) and (b), vertical lines show s.e.mean.

Bath application of dephostatin (50 μM) also increased calcium channel currents evoked by a 20 ms step to +10 mV from a holding potential of −60 mV, from 35±6 pA to 53.4±9 pA (52±10% increase), ($n = 4$, $P < 0.02$, Figure 7). Although it increased the magnitude of the currents evoked by depolarization, dephostatin did not affect the voltage at which the peak of the I-V curve is evoked. The effect of dephostatin was completely abolished by pre-incubating the cells with 100 μM tyrphostin-23 ($n = 4$, Figure 8).

To confirm that PV, the most effective phosphatase inhibitor at increasing calcium channel currents in these studies, increased cellular tyrosine phosphorylation, a crude cell lysate of rabbit ear artery cells was analysed by Western blotting. Western blotting with a specific anti-phosphotyrosine antibody, following SDS gel electrophoresis, showed a number of tyrosine phosphorylated proteins in control and PV treated cells (Figure 9a). Densitometric analysis of the blot showed a consistent increase in tyrosine phosphorylation of endogenous proteins of approximate molecular weights, 93, 87, 63.5, 45, 41.5 and 27 kDa, following pre-incubation of cells with peroxyvanadate ($n = 3$, Figure 9b).

Discussion

There is now substantial evidence indicating a widespread role for tyrosine phosphorylation in modulating ion channels in a number of cell types. Potassium channels are a target for tyrosine kinase modulation. For example the delayed rectifier-type Kv1.2 channel has been shown to be modulated by a

calcium-dependent tyrosine kinase PYK2 (Lev *et al*., 1995): Ca^{2+} (K_{Ca}) and voltage-activated (K_v) potassium channel activities were increased by activation of another tyrosine kinase JAK2 (Prevarskaya *et al*., 1995) and Src has been shown to modulate a human potassium channel (hKv1.5), probably by a direct interaction, since immunoprecipitation studies showed association of v-src with cloned and native hKv1.5 in human myocardium (Holmes *et al*., 1996). Tyrosine phosphorylation also modulates other ionic channels, including the nicotinic acetylcholine receptor channel (Siegulbaum, 1994)) as well as a γ-aminobutyric acid (GABA)-gated ion channel (Valenzuela *et al*., 1995). Recently there has been an accumulation of evidence showing calcium channel modulation in different cell types by receptor and non-receptor tyrosine kinases. IGF-1 which is linked to a receptor PTK increases VOC currents in pituitary cells (Selinfreund & Blair, 1994) and PDGF and EGF increase VOC activity in gastric mucosal cells (Slomiany *et al*., 1992; Liu *et al*., 1993). Tyrosine kinases have also been shown to modulate VOC in rat ventricular cells (Yokoshiki *et al*., 1995) and pituitary GH3 cells (Cataldi *et al*., 1996). In vascular smooth muscle cells we have shown that receptor tyrosine kinases such as the PDGF receptor (Wijetunge & Hughes, 1995a) and the non-receptor tyrosine kinase, c-Src (Wijetunge & Hughes, 1995b) increase VOC currents. In this study we present further evidence that tyrosine phosphorylation modulates VOC currents in vascular smooth muscle cells by using PTP inhibitors.

Unlike serine/threonine phosphorylated proteins, phosphotyrosine content in cells accounts for only a small percentage (0.01–0.05%) of the total phosphoamino content (Lau *et al*., 1989). It is likely that PTPs have a physiological role in maintaining this low level. There are two major groups of PTPs; the receptor-like PTPs such as the lymphocyte cell surface molecule CD45 and the non-receptor PTPs such as PTP 1B. PTPs are widely distributed in various tissues and present both in particulate and soluble fractions of cell lysates (Lau *et al*., 1989). Interestingly the specific activity of PTPs is 10–1000 times greater than that of tyrosine kinases (Fischer *et al*., 1991); therefore modulation of tyrosine phosphatase activity may represent an important, though as yet largely

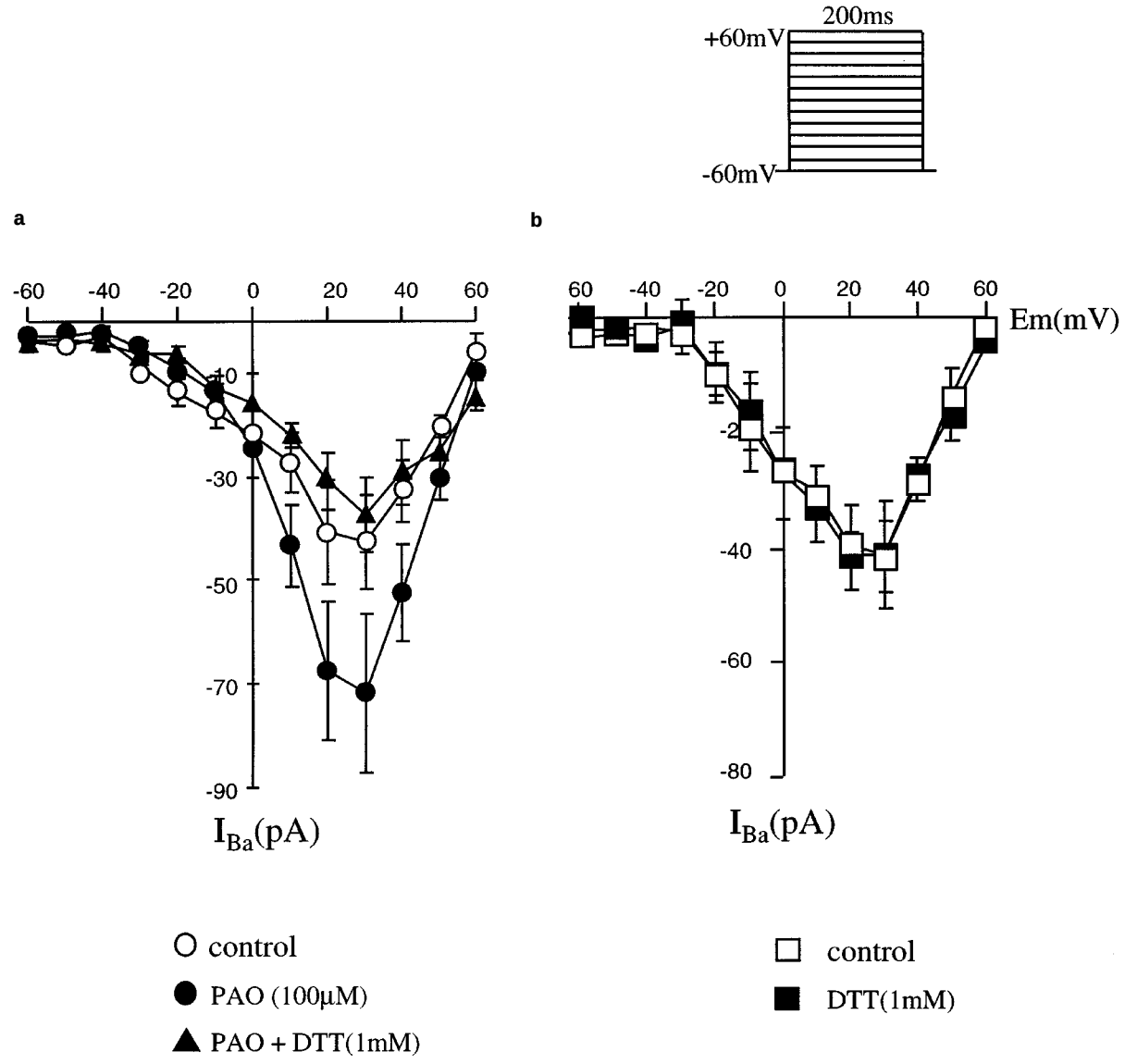

Figure 6 Effect of phenylarsine oxide (PAO) on voltage-operated calcium channel currents. PAO (100 μM) was applied to the bath solution following stabilization of control current. *I*/V relationships were derived from a holding potential of −60 mV by depolarizing to various test potentials as shown in the protocol (insert). *I*/V data were measured when the size of the calcium channel currents had stabilized before or following application of drugs. (a) Effect of PAO on the mean *I*-V relationship ($n=3-9$). (b) Effect of DTT on the mean *I*-V relationship ($n=4$). Vertical lines show s.e.mean.

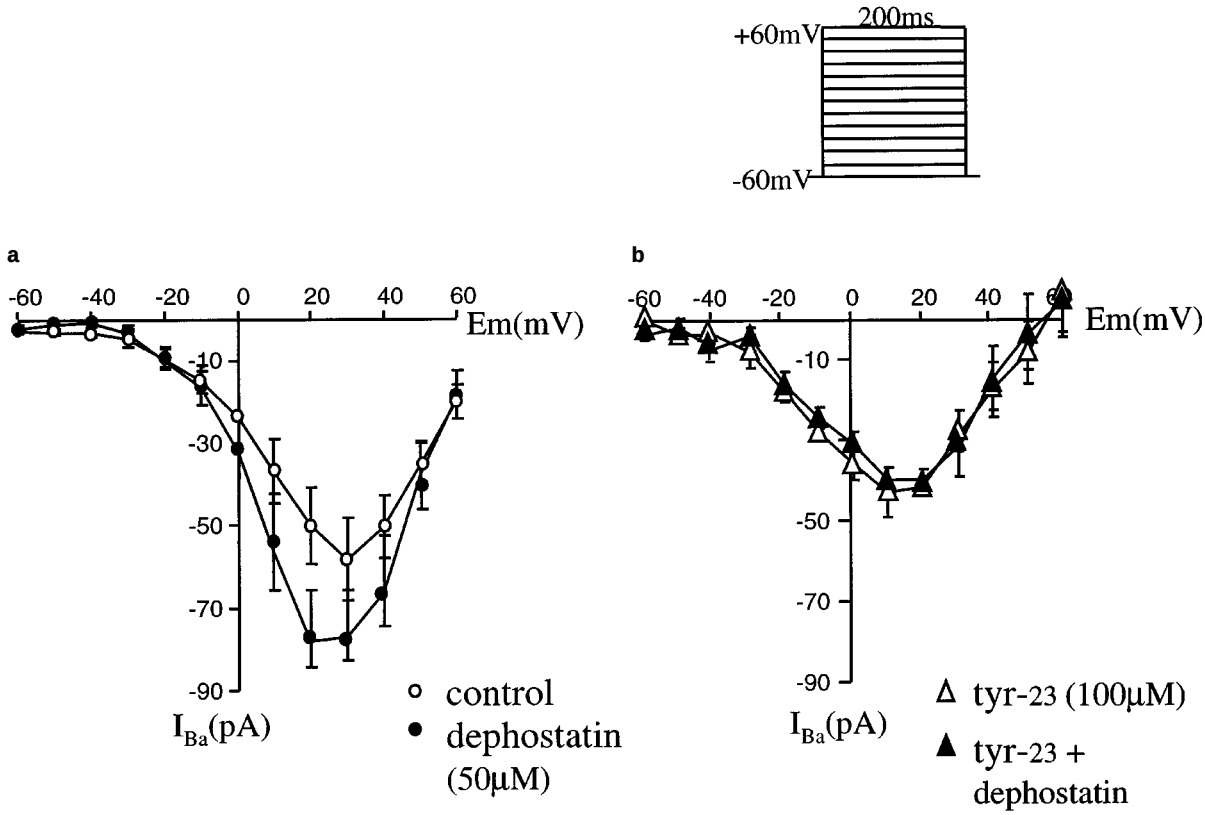

Figure 7 Effect of dephostatin on voltage-operated calcium channel currents in a rabbit single ear artery cell. Dephostatin (50 μM) was applied to the bath solution. *I*-V data were measured when the size of the calcium channel currents had stabilized. *I*-V relationships were derived from a holding potential of -60 mV by depolarizing to various test potentials as shown in the insert. (a) Effect of dephostatin on the mean *I*-V relationship ($n=4$). (b) Effect of dephostatin in the presence of 100 μM tyrphostin-23 ($n=4$). Vertical lines show s.e.mean.

unexplored physiological control mechanism within the cell. A physiological regulatory role for PTPs is suggested by the observations that *Rous sarcoma* virus transformation of fibroblasts is accompanied by a large increase in PTP activity (Nelson & Branton, 1984), and in other systems co-ordinated increases in both tyrosine kinases and PTPs have been observed (Gentleman *et al*., 1984).

Sodium orthovanadate, a potent insulinomimetic agent, is a widely used inhibitor of PTP known to cause tyrosine phosphorylation in many cell types (Roth & Cassell, 1983; Kadota *et al*., 1987). Sodium orthovanadate has been shown to cause contraction of smooth muscle associated with an increase in tyrosine phosphorylation and increased calcium entry (Di Salvo *et al*., 1993). Recently Cataldi *et al*. (1996) showed that vanadate increased voltage-operated calcium channel currents in pituitary GH3 cells. Furthermore in single smooth muscle cells of the rabbit colonic muscularis mucosae, nifedipine-sensitive L-type channels were inhibited by genistein and tyrphostin, whereas vanadate enhanced the peak current (Hatakeyama *et al*., 1996). In keeping with the above studies, in our study sodium orthovanadate increased VOC currents in rabbit ear artery smooth muscle cells. Hydrogen peroxide (H_2O_2), an active oxygen species and a cellular oxidant, is also an insulinomimetic agent and a PTP inhibitor known to stimulate tyrosine phosphorylation in cells (Kadota *et al*., 1987). It has been found that a combination of H_2O_2 with orthovanadate which forms peroxyvanadate (PV) increases its ability to act as an inhibitor of PTP (Kadota *et al*., 1987). Consistent with this finding, PV increased calcium channel currents approximately 1.5 times more than vanadate alone. This observation is in keeping with functional studies by Laniyonu *et al*. (1994), who previously showed that PV caused vascular smooth muscle contraction via a mechanism inhibited by calcium channel antagonists. However, despite these findings being consistent with PTP modulation of VOC, vanadate compounds are not selective for inhibition of PTPs and are known to inhibit a number of other enzymes, such as Na^+/K^+-ATPase and Ca^{2+}-ATPase (Nechay, 1984). H_2O_2 also may have other PTP-independent actions. It has been shown that PDGF-induced tyrosine phosphorylation, mitogenesis and chemotaxis require generation of H_2O_2, suggesting that H_2O_2 itself could act as a signal transducting molecule in some systems (Sundaresan *et al*., 1995). Therefore we used a selective tyrosine kinase inhibitor, tyrphostin-23 to confirm that the actions of PV were mediated by increased tyrosine phosphorylation.

Tyrphostin-23 is a low molecular weight tyrosine kinase inhibitor known to inhibit selectively tyrosine kinases in a number of systems by binding to the substrate binding site of tyrosine kinases (Gazit *et al*., 1989). In our previous studies tyrphostin-23 reduced calcium channel currents in the absence of exogenous stimulation (Wijetunge *et al*., 1992) and also inhibited the effects of PDGF and c-Src (Wijetunge & Hughes, 1995a,b) without inhibiting the actions of a dihydropyridine calcium channel agonist (Wijetunge & Hughes, 1995a). In this study PV-induced increase in calcium channel currents were completely blocked by tyrphostin-23. This indicates that the increase in calcium channel currents was probably due to increased tyrosine phosphorylation. Using a similar approach Laniyonu *et al*. (1994) also concluded that the VOC-dependent

smooth muscle contractions were linked to a tyrosine kinase signalling pathway and not related to inhibition of Na^+/K^+-ATPase or Ca^{2+}-ATPase.

We have provided evidence that phosphatase inhibitors increase cellular tyrosine phosphorylation by showing that pre-incubation with peroxyvanadate, consistently increased tyrosine phosphorylation of a number of endogenous proteins in rabbit differentiated ear artery cells. This result is consistent with those of Laniyonu *et al.* (1994), where PV caused a marked increase in aortic and gastric tissue tyrosine phosphorylation which was inhibited by genistein or tyrphostin.

Our studies were further extended by the use of two other structurally unrelated inhibitors of PTP, phenylarsine oxide (PAO) and dephostatin. PAO is a membrane permeable PTP inhibitor which selectively augments tyrosine phosphorylation without affecting the serine/threonine phosphate content in cells (Oetken *et al.*, 1992). It is thought that PAO acts by a direct oxidative mechanism involving the catalytic site of PTP which contains an essential cysteine residue (Fischer *et al.*, 1991), and reducing agents such as DTT are known to reverse the inhibitory action of PAO on PTP (Hecht & Zick, 1992). In rabbit ear artery cells, PAO increased VOC currents in a manner similar to that seen following vanadate or PV. This action of PAO was reversed by DTT, consistent with its mechanism of action involving inhibition of PTPs. It has been shown that thiol compounds are required for the activity of some PTPs (Shriner & Brautigan, 1984; Tonks *et al.*, 1988). DTT itself has been shown to stimulate a bovine PTP activity when added to the assay buffer (Lau *et al.*, 1987) and hence might be anticipated to reduce calcium channel currents.

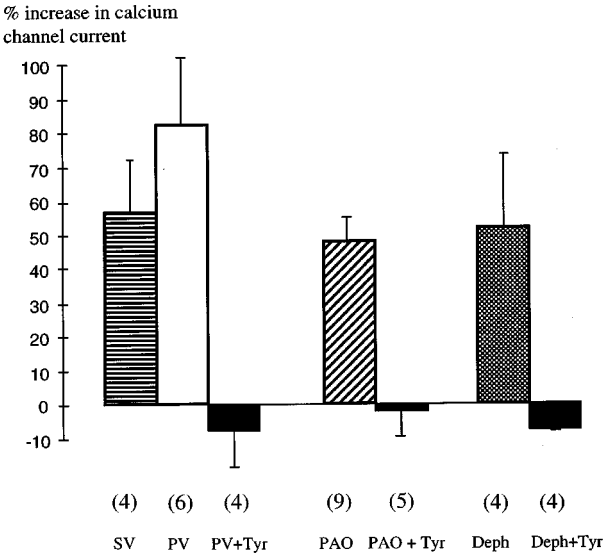

Figure 8 Effects of PTP inhibitors in the presence and absence of tyrphostin-23 (Tyr) on voltage-operated calcium channel currents in rabbit ear artery cells. Currents were evoked by a 20 ms depolarization to +10 mV from a holding potential of −60 mV. Figure shows % increase in peak current induced by the PTP inhibitors. Sodium orthovanadate (SV) and peroxyvanadate (PV) were included in the pipette solution, whereas PAO and dephosatatin (Deph) were applied to the bath solution. Tyrphostin (100 μM) was added to the bath solution and the cells were pre-incubated in this solution for at least 10 min before the establishment of whole cell mode with intracellular pipette containing PV or addition of PAO or dephostatin to the bath. For SV and PV, % increases were calculated by measuring the curents 5–6 min following establishment of whole cell mode, when currents had reached a steady level, compared with currents measured 10 s after establishment of whole cell mode. For PAO and dephostatin % increases were calculated by measuring the stable currents before and after bath application of the drugs. Columns represent means±s.e.mean of (n) observations.

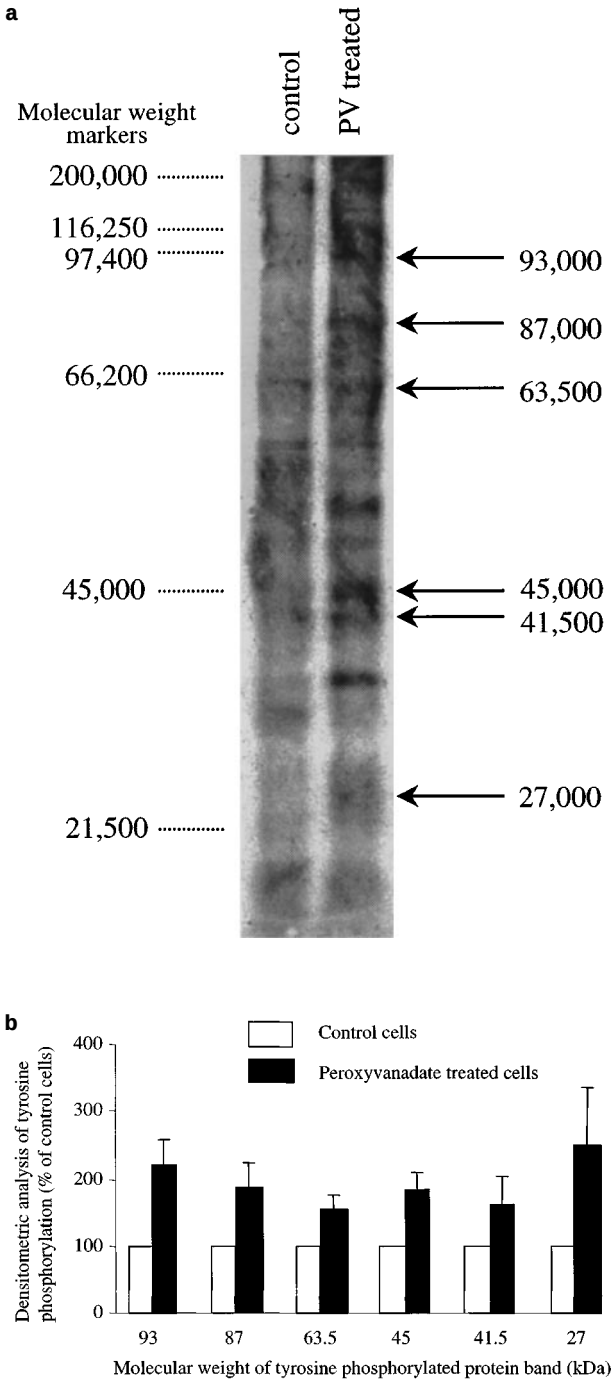

Figure 9 Tyrosine phosphorylation profile of rabbit ear artery cell lysates in the presence and absence of peroxyvanadate. (a) Cell lysate proteins (15 μg) from control and peroxyvanadate-treated cells were separated on a 10% polyacrylamide gel and immunoblotted with an antiphosphotyrosine antibody (PY20). Proteins were detected with an HRP-linked secondary antibody followed by enhanced chemiluminescence. The data shown are representative of 3 separate experiments. Molecular weights as determined by biotinylated molecular weight markers are indicated on the left and approximate molecular weights of the proteins which show enhanced tyrosine phosphorylation are indicated by arrows on the right. (b) Densitometric analysis of the tyrosine phosphorylated bands. The effect of peroxyvanadate on the tyrosine phosphorylation, of bands corresponding to 93, 87, 63.5, 45, 41.5 and 27 kDa, is shown. Tyrosine phosphorylation of these protein bands in control cells was normalized to 100%. The results are means±s.e.mean of 3 separate experiments from 3 different rabbit ear artery cell preparations.

However, in our study DTT on its own did not have any significant effects on calcium channel currents.

Dephostatin is a novel PTP inhibitor isolated from the culture broth of a strain of *Streptomyces* and is thought to act by competing with the substrate (Imoto *et al*., 1993). It contains a unique N-nitrosamine function and inhibits *Yersinia* and mammalian PTP activities (Yu *et al*., 1995). Dephostatin also increased VOC currents and the effects of both PAO and dephostatin were blocked by tyrpostin-23 indicating that the action of these agents involved tyrosine phosphorylation.

Our results collectively suggest that there is basal activity of PTP in vascular smooth muscle and that inhibiting tyrosine phosphatases, increases calcium channel currents under resting conditions by increasing overall levels of cellular tyrosine phosphorylation. Vascular smooth muscle cells contain both L-type and T-type calcium channels (reivewed in Hughes, 1995). Although it has been shown that T-type channels are regulated by tyrosine phosphorylation in fibroblasts (Wang *et al*., 1993), in rabbit ear artery cells the effects of tyrosine phosphorylation are likely to be due mainly to an effect on L-type channels since T-type channel currents were almost undetectable in these cells. The mechanism by which tyrosine phosphorylation increases L-type calcium channel currents in this study is not known. The channel protein may be directly phosphorylated on tyrosine residues, as has been observed in gastric mucosal cell calcium channel subunits of 170 and 55 kDa in response to PDGF and EGF (Slomiany *et al*., 1992; Liu *et al*., 1993). Direct tyrosine phosphorylation-dependent modulation has also been seen in a nicotinic acetylcholine receptor channel and a delayed rectifier voltage-dependent potassium channel (Siegelbaum, 1994). Alternatively tyrosine phosphorylation may modulate VOC by activating a second messenger system. Although activation of PKC is known to increase calcium channel currents in vascular smooth muscle (Loirand *et al*., 1990), we have previously ruled out the possibility of PKC being a second messenger in rabbit ear artery cells in response to PDGF (unpublished data) or c-Src (Wijetunge & Hughes, 1995), since inhibition of PKC by a selective PKC inhibitory peptide did not affect the response to PDGF or c-Src. However, we cannot at present rule out one of the other several second messenger systems activated by PTK from involvement in these effects.

In conclusion, our results suggest that the overall levels of tyrosine phosphorylation in vascular smooth muscle cells are an important influence modulating the opening of VOC. Regulation of either tyrosine kinase or tyrosine phosphatase activity may represent novel approaches to influencing calcium entry through voltage-operated calcium channels.

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References

AARONSON, P.I., BOLTON, T.B., LANG, R.J. & MACKENZIE, I. (1988). Calcium currents in single isolated smooth muscle cells from the rabbit ear artery in normal calcium and high barium solutions. *J. Physiol.,* **405,** 57 – 75.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol.,* **381,** 385 – 406.

BENHAM, C.D. & TSIEN, R.W. (1988). Noradrenaline modulation of calcium channels in single smooth muscle cells from rabbit ear artery. *J. Physiol.,* **404,** 767 – 784.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 – 254.

CATALDI, M., TAGLIALATELA, M., GUERRIERO, S., AMOROSO, S., LOMBARDI, G., DI RENZO, G. & ANNUNZIATO, L. (1996). Protein-tyrosine kinases activate while protein-tyrosine phosphatases inhibit L-type calcium channel activity in pituitary GH3 cells. *J. Biol. Chem.,* **271,** 9441 – 9446.

COURTNEIDGE, S.A. (1994). Protein tyrosine kinases, with emphasis on the Src family. *Sem. Cancer Biol.,* **5,** 239 – 246.

DI SALVO, J., GIFFORD, D. & KOKKINAKIS, A. (1989). ATP- and polyphosphate-mediated stimulation of pp60c-src kinase activity in extracts from vascular smooth muscle. *J. Biol. Chem.,* **264,** 10773 – 10778.

DI SALVO, J., SEMENCHUK, L.A. & LAUER, J. (1993). Vanadate-induced contraction of smooth muscle and enhanced protein tyrosine phosphorylation. *Arch. Biochem. Biophys.,* **304,** 386 – 391.

FISCHER, E.H., CHARBONNEAU, H. & TONKS, N.K. (1991). Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. *Science,* **253,** 401 – 407.

FUKUMITSU, T., HAYASHI, H., TOKUNO, H. & TOMITA, T. (1990). Increase in calcium channel current by β-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery. *Br. J. Pharmacol.,* **100,** 593 – 599.

GAZIT, A., YAISH, P., GILON, C. & LEVITZKI, A. (1989). Tyrphostins 1: synthesis and biological activity of protein tyrosine kinase inhibitors. *J. Med. Chem.,* **32,** 2344 – 2352.

GENTLEMAN, S., MARTENSEN, T.M., DIGIOVANNA, J.J. & CHADER, D.J. (1984). Protein tyrosine kinase and protein phosphotyrosine phosphatase in normal and psoriatic skin. *Biochim. Biophys. Acta,* **798,** 53 – 59.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch clamp techniques for high resolution current recordings from cells and cell free membrane patches. *Pflügers Arch.,* **391,** 85 – 100.

HATAKEYAMA, N., MUKHOPADHYAY, D., GOYAL, R.K. & AKBAR-ALI, H.I. (1996). Tyrosine kinase-dependent modulation of calcium entry in rabbit colinic muscularis mucosae. *Am. J. Physiol.,* **270,** C1780 – C1789.

HECHT, D. & ZICK, Y. (1992). Selective inhibition of protein tyrosine phosphatase activities by H202 and vanadate in vitro. *Biochem. Biophys. Res. Commun.,* **188,** 773 – 779.

HERING, S., KLEPPISCH, T., TIMIN, E.N. & BODEWEI, R. (1989). Characterisation of the calcium channel state transitions induced by the enantiomers of the 1,4-dihydropyridine Sandoz 202-791 in neonatal rat heart cells. A nonmodulated receptor model. *Pflügers Arch.,* **414,** 690 – 700.

HOLLENBERG, M.D. (1994). Tyrosine kinase pathways and the regulation of smooth muscle contractility. *Trends Pharmacol. Sci.,* **15,** 108 – 114.

HOLMES, T.C., FADOOL, D.A., REN, R.B. & LEVITAN, I.B. (1996). Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. *Science,* **274,** 2089 – 2091.

HUGHES, A.D. (1995). Calcium channels in vascular smooth muscle cells. *J. Vasc. Res.,* **32,** 353 – 370.

IMOTO, M., KAKEYA, H., SAWA, T., HAYASHI, C., HAMADA, M., TAKEUCHI, T. & UMEZAWA, K. (1993). Dephostatin, a novel protein tyrosine phosphatase inhibitor produced by Streptomyces. *J. Antiobiot.,* **46,** 1342 – 1346.

ISHIDA, M., MARRERO, M.B., SCHEIFFER, B., ISHIDA, T.. BERNSTEIN, K.E. & BERK, B.C. (1995). Angiotensin II activates pp60c-src in vascular smooth muscle cells. *Circ. Res.,* **77,** 1053 – 1059.

KADOTA, S., FANTUS, I.G., DERAGON, G., GUYDA, H.J., HERSH, B. & POSNER, B.I. (1987). Peroxides of vanadium: a novel and potent insulin-mimetic agent which activates the insulin receptor kinase. *Biochem. Biophys. Res. Commun.,* **147,** 259 – 266.

LANIYONU, A., SAIFEDDINE, M., AHMED, S. & HOLLENBERG, M.D. (1994). Regulation of vascular and gastric smooth muscle contractility by pervanadate. *Br. J. Pharmacol.,* **113,** 403 – 410.

LAU, K.H.W., FARLEY, J.R. & BAYLINK, D.J. (1989). Phosphotyrosyl protein phosphatases. *Biochem. J.,* **257,** 23 – 36.

LAU, K.H.W., FREEMAN, T.K. & BAYLINK, D.J. (1987). Purification and characterisation of an acid phosphatase that displays phosphotyrosyl-protein phosphatase activity from bovine cortical bone matrix. *J. Biol. Chem.,* **262,** 1389 – 1397.

LEV, S., MORENO, H., MARTINEZ, R., CANOLL, P., PELES, E., MUSACCHIO, J.M., PLOWMAN, G.D., RUDY, B. & SCHLESSINGER, J. (1995). Protein tyrosine kinase PYK2 involved in Ca2 + -induced regulation of ion channel and MAP kinase functions. *Nature,* **376,** 737 – 745.

LIU, X., BRODEUR, S.R., GISH, G., SONGYANG, Z., CANTLEY, L.C., LAUDANO, A.P. & PAWSON, T. (1993). Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene,* **8,** 1119 – 1126.

LIU, J., FEKETE, Z., SLOMIANY, A. & SLOMIANY, B.L. (1993). Activation of gastric mucosal calcium channels by epidermal growth factor. *J. Biochem.,* **25,** 29 – 35.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1990). GTP-binding proteins mediate noradrenaline effects on calcium and chloride currents in rat portal vein myocytes. *J. Physiol.,* **428,** 517 – 529.

MALIK, R.J. & PARSONS, J.T. (1996). Integrin-mediated signalling in normal and malignant cells: a role of protein tyrosine kinases. *Biochim. Biophys. Acta,* **1287,** 73 – 76.

NECHAY, B.R. (1984). Mechanisms of action of vanadium. *Annu. Rev. Pharmacol. Toxicol.,* **24,** 501 – 524.

NELSON, R.L. & BRANTON, P.E. (1984). Identification, purification, and characterisation of phosphotyrosine-specific protein phosphatases from cultured chicken embryo fibroblasts. *Mol. Cell. Biol.,* **4,** 1003 – 1012.

OETKEN, C., VON WILLEBRAND, M., AUTERO, M., RUUTU, T., ANDERSSON, L.C. & MUSTELIN, T. (1992). Phenylarsine oxide augments tyrosine phosporylation in hematopoietic cells. *Eur. J. Haematol.,* **49,** 208 – 214.

PREVARSKAYA, N.B., SKRYMA, R.N., VACHER, P., DANIEL, N., DJIANE, J. & DUFY, B. (1995). Role of tyrosine phosphorylation in potassium channel activation. *J. Biol. Chem.,* **270,** 24292 – 24299.

ROTH, R.A. & CASSELL, D.J. (1983). Insulin receptor: evidence that it is a protein kinase. *Science,* **219,** 299 – 301.

SCHLESSINGER, J. & ULLRICH, A. (1992). Growth factor signalling by receptor tyrosine kinases. *Neuron,* **61,** 383 – 391.

SELINFREUND, R.H. & BLAIR, L.A.C. (1994). Insulin-like growth factor-1 induces a rapid increase in calcium currents and spontaneous membrane activity in clonal pitiutary cells. *Mol. Pharmacol.,* **45,** 1215 – 1220.

SHRINER, C.L. & BRAUTIGAN, D.L. (1984). Cytosolic protein phosphotyrosine phosphatases from rabbit kidney. Purification of two distinct enzymes that bind to Zn2 + -iminodiacetate agarose. *J. Biol. Chem.,* **259,** 11383 – 11390.

SIEGELBAUM, S.A. (1994). Ion channel control by tyrosine phosphorylation. *Current Biol.,* **4,** 242 – 245.

SIMONSON, M.S. & HERMAN, W.H. (1993). Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signalling by endothelin-1. Cross-talk between G protein-coupled receptors and pp60c-src. *J. Biol. Chem.,* **268,** 9347 – 9357.

SLOMIANY, B.L., LIU, J. & SLOMIANY, A. (1992). Platelet-derived growth factor activation of gastric mucosal calcium channels. *Biochem. Biophys. Res. Commun.,* **186,** 1496 – 1502.

SUNDARESAN, M., YU, Z-X., FERRANS, V.J., IRANI, K. & FINKEL, T. (1995). Requirement for generation of H202 for platelet-derived growth factor signal transduction. *Science,* **270,** 296 – 299.

TEWARI, K. & SIMRAD, J.M. (1994). Protien kinase A increases availability of calcium channels in smooth muscle cells from guinea pig basilar artery. *Pflügers Arch.,* **428,** 9 – 16.

TONKS, N.K., DILTZ, C.D. & FISCHER, E.H. (1988). Characterization of the major tyrosine phosphatases of human placenta. *J. Biol. Chem.,* **263,** 6731 – 6737.

TOYOSHIMA, K., YAMANASHI, Y., INOUE, K., SEMBA, K., YAMAMOTO, T. & AKIYAMA, T. (1992). Protein tyrosine kinases belonging to the Src family. *Ciba Found. Symp.,* **164,** 240 – 248.

VALENZUELA, C.F., MACHU, T.K., MCKERNAN, R.M., WHITING, P., VANRENTERGHEM, B.B., MCMANAMAN, J.L., BROZOWSKI, S.J., SMITH, G.B., OLSEN, R.W. & HARRIS, R.A. (1995). Tyrosine kinase phosphorylation of $GABA_A$ receptors. *Brain Res. Mol. Brain Res.,* **31,** 165 – 172.

WANG, Z., ESTACION, M. & MORDAN, L.J. (1993). Influx via T-type calcium channels modulates PDGF-induced replication of mouse fibroblasts. *Am. J. Physiol.,* **265,** C1239 – C1246.

WIJETUNGE, S., AALKJAER, C., SCHACHTER, M. & HUGHES, A.D. (1992). Tyrosine kinase inhibitors block calcium currents in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **189,** 1620 – 1623.

WIJETUNGE, S. & HUGHES, A.D. (1995a). Effect of platelet-derived growth factor on voltage-operated calcium channels in rabbit isolated ear arteries. *Br. J. Pharmacol.,* **115,** 534 – 538.

WIJETUNGE, S. & HUGHES, A.D. (1995b). pp60c-src increases voltage-operated calcium channel currents in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **217,** 1039 – 1044.

WIJETUNGE, S. & HUGHES, A.D. (1996). Activation of endogenous c-Src or a related tyrosine kinase by intracellular (pY)EEI peptide increases voltage-operated calcium channel currents in rabbit ear artery cells. *FEBS. Lett.,* **399,** 63 – 66.

YOKOSHIKI, H., SUMII, K. & SPERILAKIS, N. (1995). Inhibition of L-type calcium current in rat ventricular cells by the tyrosine kinase inhibitor, Genistein and its inactive analogue, Diadzein. *J. Mol. Cell. Cardiol.,* **28,** 807 – 814.

YU, L., MCGILL, A., RAMIREZ, J., WANG, P.G. & ZHANG, Z. (1995). Synthesis and bioassay of a protein tyrosine phosphatase inhibitor, Dephostatin. *Bioorg. Mel. Chem. Lett.,* **5,** 1003 – 1006.

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