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# Involvement of tyrosine phosphorylation in endothelin-1-induced calcium-sensitization in rat small mesenteric arteries

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**1** We have studied the effect of endothelin-1 stimulation on protein tyrosine phosphorylation levels in intact small mesenteric arteries of the rat and investigated the effects of tyrosine kinase inhibition on the contractile response to this agonist.

**2** Endothelin-1 stimulated a rapid (20 s), sustained (up to 20 min) and concentration-dependent (1–100 nM) increase in protein tyrosine phosphorylation levels which coincided temporally with the contractile response in intact and α-toxin permeabilized small artery preparations. Tyrosine phosphorylation was increased in four main clusters of proteins of apparent molecular mass 28–33, 56–61, 75–85 and 105–115 kDa. Endothelin-1-induced protein tyrosine phosphorylation was independent of extracellular calcium, antagonized by the tyrosine kinase inhibitor tyrphostin A23 but not by the inactive tyrphostin A1.

**3** In intact small arteries tyrphostin A23 inhibited the force developed to endothelin-1 at all concentrations studied; at higher concentrations (10 and 100 nM) the profile of contraction was altered from a sustained to a transient response. Tyrphostin A1 inhibited the contractile response to endothelin-1 at all concentrations except 100 nM; the profile of the response was not altered. Neither tyrphostin affected the transient phasic contraction induced by endothelin-1 (100 nM) in the absence of extracellular calcium.

**4** In rat α-toxin permeabilized mesenteric arteries endothelin-1 caused a concentration-dependent increase in force in the presence of 10 μM GTP and low (pCa 6.7) constant calcium, demonstrating increased sensitivity of the contractile apparatus to calcium. Tyrphostin A23 inhibited this response by approximately 50%, tyrphostin A1 did not affect endothelin-1-induced calcium sensitization of force.

**5** We conclude that increased tyrosine phosphorylation is important in the contractile response induced by endothelin-1 in intact small mesenteric arteries. Furthermore our data implicate activation of this signalling pathway in the tonic phase of contraction possibly through modulation of the sensitivity of the contractile apparatus to calcium.

**Keywords:** Endothelin-1; tyrosine phosphorylation; calcium sensitization; vascular smooth muscle; rat mesenteric arteries

## Introduction

Endothelin-1 (ET-1) is a member of a family of potent vasoconstrictor peptides first isolated from the supernatant of porcine cultured endothelial cells (Yanagisawa *et al*., 1988). Three endothelins have been identified, ET-1, ET-2 and ET-3 (Inoue *et al*., 1989) and two endothelin receptors $ET_A$ and $ET_B$ are known to mediate their effects (Arai *et al*., 1990; Sakurai *et al*., 1990). In the rat isolated perfused mesentery $ET_A$ receptors mediate the vasoconstrictor response to ET-1 whilst $ET_B$ receptors mediate endothelium-dependent dilatation (Warner *et al*., 1993). However, in isolated segments of rat mesenteric arteries the receptors which mediate the endothelium-dependent relaxation to ET-1, presumably $ET_B$ receptors, appear not to be present (Hiley & Poon, 1994).

Contraction of smooth muscle in response to ET-1 was originally proposed to be mediated by $Ca^{2+}$ influx through voltage-dependent calcium channels (Goto *et al*., 1989). However, further studies have shown that there are three principal mechanisms by which ET-1 elevates intracellular $Ca^{2+}$. (1) release of $Ca^{2+}$ from intracellular stores secondary to inositol trisphosphate production, (2) activation of voltage-dependent $Ca^{2+}$ channels and (3) $Ca^{2+}$ entry via other types of channels (Simonson & Dunn, 1990; Highsmith *et al*., 1992). The mechanism employed appears to be cell type- and concentration-dependent (Sakata *et al*., 1989; Xuan *et al*., 1994; Enoki *et al*., 1995). However, the contractile response of vascular smooth muscle to ET-1 is not dependent solely on elevated $Ca^{2+}$ levels. Complete inhibition of ET-1 stimulated $Ca^{2+}$ elevation by nicardipine only partially suppressed the contractile response in rat aorta (Sakata *et al*., 1989), suggesting that ET-1 increased the sensitivity of the contractile apparatus to $Ca^{2+}$ – an observation confirmed by Nishimura *et al*. (1992) in rabbit α-toxin permeabilised mesenteric arteries, a preparation which allows intracellular $Ca^{2+}$ levels to be controlled but leaves receptors coupled to their intracellular signalling pathways (Nishimura *et al*., 1988; Kitazawa *et al*., 1989). In this preparation ET-1 induced a marked enhancement of force in the presence of a low constant level of $Ca^{2+}$, which was dependent on guanosine 5′-trisphosphate (GTP) implying a role for G proteins in the effect (Nishimura *et al*., 1992). However, the mechanisms involved in ET-1 induced $Ca^{2+}$ sensitization are unclear.

Endothelin-1 activates multiple signalling systems in vascular smooth muscle, involving such effectors as phospholipase C (PLC), phospholipase D (PLD), phospholipase $A_2$ ($PLA_2$) and protein kinase (PKC) (reviewed Simonson & Dunn, 1990). In addition, increased phosphorylation of proteins on tyrosine residues following ET-1 stimulation has been observed in vascular smooth muscle cells, implicating tyrosine kinases and/or phosphatases in the responses to this peptide (Koide *et al*., 1992). Initially the role of tyrosine kinases in cell signalling was thought to be restricted to long term effects such as growth and proliferation. However, it is now recognized that increased protein tyrosine phosphorylation occurs rapidly (within seconds) in response to both polypeptide growth factors and vasoconstrictor hormones (Tsuda *et al*., 1991; Zachary *et al*., 1992; Abedi *et al*., 1995). Furthermore, the observation that epidermal growth factor and platelet derived growth factor increase vascular tone (Hollenberg, 1994; Hughes, 1995), an effect blocked by genistein and tyrphostins, both inhibitors of

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tyrosine kinases, supports the possibility that tyrosine phosphorylation is involved in the contractile response (reviewed Hollenberg, 1994). Previously we have shown in rat mesenteric small arteries that the vasoconstrictor hormone noradrenaline increases tyrosine phosphorylation (Ward *et al*., 1995), and recently it was demonstrated that tyrosine kinase inhibitors reversibly antagonized the contractile response to this agonist in the same preparation (Toma *et al*., 1995). However, whether other vasoconstrictor agonists utilise this signalling pathway to mediate the contractile response is not known.

In this study, we have investigated the mechanisms that regulate protein tyrosine phosphorylation in rat mesenteric small arteries following ET-1 stimulation. We have combined these studies with functional measurements of contraction in intact and α-toxin permeabilized arterial segments, and present evidence that increased protein tyrosine phosphorylation is important for the sustained phase of contraction in response to ET-1.

## Methods

### *Preparation of small arteries*

Adult female Sprague Dawley rats (200–300 g) were killed by stunning and cervical dislocation. The mesentery was immediately excised and kept in ice-cold physiological salt solution (PSS) ((mM) NaCl 119, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.17, $KH_2PO_4$ 1.18, $K_2$ EDTA 0.026, glucose 5.5 and $CaCl_2.2H_2O$ 2.5; pH 7.2) until dissection. Mesenteric small arteries (second, third and fourth order branches) $<300$ μm internal diameter were cleaned of adjoining fat and connective tissues before use in all subsequent stages.

### *Phosphotyrosine immunoblots*

The method used was essentially as described previously (Ward *et al*., 1995). Briefly, following dissection vessels were incubated at 37°C in 100 μl of culture medium M199 containing (major constituents only) the following (mM): NaCl 128, KCl 5.4, $MgSO_4.7H_2O$ 0.34, $CaCl_2.2H_2O$ 1.3, $NaHCO_3$ 4.2, $KH_2PO_4$ 0.44, $Fe(NO_3)_3.9H_2O$ 0.002, glucose 5.6 and HEPES 25, for 30 min. Arteries were stimulated by the addition of 100 μl of ET-1 at twice the final concentration in prewarmed M199. Final concentrations and times of treatment were as described in individual figure legends. The reaction was terminated by the rapid transfer of tissues into 110 μl of ice-cold homogenisation buffer ((mM) Tris 20, sucrose 250, EDTA 5, EGTA 10 and dithiothreitol 10; pH 7.5, containing the following phosphatase and protease inhibitors: 1 mM sodium orthovanadate, 200 μM sodium pyrophosphate, 1 μM $ZnCl_2$, 1 mM phenylmethanesulphonyl fluoride and 100 μM leupeptin) and homogenized in a hand held ground glass homogenizer. The sample was centrifuged at 12,000 $\times g$ for 10 s to remove nuclei, cell debris and non-homogenized tissue; 60 μl of supernatant was removed and a 10 μl aliquot reserved for protein estimation. The remaining homogenate was mixed with 5 fold-concentration of Laemmli sample buffer, heated at 100°C for 3 min and resolved by SDS–PAGE. Equal amounts of protein, 15 μg were loaded for each sample. After electrophoresis, proteins were electrophoretically transferred to Hybond-C by the method of Towbin *et al*. (1979) and processed for Western blot analysis. The primary antibody was antiphosphotyrosine antibody (RC20) coupled to horseradish peroxidase. Signals were developed by use of an enhanced chemiluminescent kit. In a subset of experiments, membranes were stripped by the manufacturer's recommended procedure (ECL, Amersham, U.K.), washed with 0.1% Tween TBS pH 8.4 (15 mM Tris, 150 mM NaCl) and reprobed with a different anti-phosphotyrosine antibody (PY54) followed by horseradish peroxidase conjugated mouse secondary antibody. Signals were developed as above. Subsequently, membranes were stained with 0.1% India ink to confirm equivalent protein loadings. Protein was estimated by the BioRad assay kit (Bradford, 1976).

The Western blot autoradiographs were scanned with a laser densitometer (Pharmacia, LKB, U.K.). The area under the curve for individual bands was used as a measure of signal intensity and analysed by Gelscan XL software (Pharmacia, LKB, U.K.) The data shown are from the 75–85 kDa band only as this cluster of proteins showed the most reproducible changes following ET-1 stimulation. The values plotted are relative intensity where basal intensity = 1. The basal value was taken as time 0 min or concentration 0 nM for each individual experiment.

### *Contractile studies*

*Intact artery preparation* Segments of mesenteric small arteries 2 mm in length were mounted as ring preparations on wires in a myograph, maintained at 37°C in PSS gassed with 95% $O_2$/5% $CO_2$ for 1 h then set to an internal circumference at which they are held just under tension (Ohanian *et al*., 1993). Cumulative concentration-response curves to ET-1 and KCl were constructed in the presence or absence of tyrphostins A1 and A23 (100 μM). Potassium solutions of the required concentrations were made by molar substitution of KCl for NaCl in PSS (K-PSS). In experiments where the effects of removing extracellular calcium were studied, $Ca^{2+}$-free PSS was made by not adding $CaCl_2$ but adding 0.1 or 2 mM EGTA. Vessels were incubated in $Ca^{2+}$-free PSS for 10 min before stimulation. Control concentration-responses were carried out in the presence of vehicle (0.1% DMSO).

*Permeabilized artery preparation* α-Toxin permeabilization of rat mesenteric small arteries was as previously described (Jensen *et al*., 1996). Briefly, mounted vessel segments were equilibrated in normal external solution ((mM) NaCl 150, KCl 4, calcium methane sulphonate 2, magnesium methane sulphonate 1, HEPES 5 and glucose 5.6, pH was adjusted to 7.4 with Tris base) for 10 min followed by stimulation with 125 mM $K^+$ solution and 10 μM noradrenaline. All solutions were gassed with 100% $O_2$ and the preparations maintained at room temperature.

Subsequently arteries were incubated in relaxing solution ((mM) PIPES 30, sodium creatine phosphate 10, $Na_2ATP$ 5.16, magnesium methane sulphonate 7.31, potassium methane sulphonate 74.1, $K_2EGTA$ 1, pH was adjustable to 7.1 with KOH) for 15 min after which they were permeabilized according to the method of Kitazawa *et al*. (1989) with minor modifications. A 10 μl droplet of pCa 6.7 solution containing 1,250 units of *Staphylococcus aureus* α-toxin and 10 μM A23187 (calcium ionophore) was placed on each vessel segment. After tension development had reached a plateau, 15–20 min, the α-toxin was washed from the myograph bath and the vessels equilibrated in relaxing solution for 20 min. In the α-toxin or activating solutions, 10 mM EGTA was used, and a specified amount of calcium methane sulphonate was added to give the desired concentration of free $Ca^{2+}$ ions (Horiuti, 1988).

The tension developed to a high concentration of $Ca^{2+}$ (pCa 5.0) was recorded. After re-equilibration in relaxing solutions, a submaximal $Ca^{2+}$ concentration (pCa 6.7) was added and a concentration-response to ET-1 in the presence of 10 μM GTP constructed. Tension was allowed to plateau before the next concentration of ET-1 was added.

The contractile response was measured when tension had reached a plateau. The data are expressed as active tension, mN $mm^{-1}$.

### *Materials*

All materials were purchased from Sigma Chemical Co., except for M199 and *Staphylococcus aureus* α-toxin (GIBCO, BRL, Paisley, U.K.), the Western blotting kit (Amersham

International, Bucks, U.K.) and tyrphostins A1 and A23 (Novabiochem/Calbiochem, Nottingham, U.K.). Antiphosphotyrosine antibodies RC20 and PY54 were purchased from AFFINITI Research Products Ltd (Nottingham, U.K.).

## Results

### *The effect of endothelin-1 on phosphotyrosine levels*

There was an increase in protein phosphotyrosine levels in homogenates of rat mesenteric small arteries following ET-1 stimulation (Figure 1a and b). Tyrosine phosphorylation was increased in proteins of apparent molecular mass 28–33 kDa, 56–61 kDa, 75–85 kDa and 105–115 kDa when the antiphosphotyrosine antibody RC20 was used (Figure 1a). Reprobing the same membrane with a second antiphosphotyrosine antibody (PY54) demonstrated slight differences. A doublet was apparent in the 75–85 kDa proteins, a stronger signal at 105–115 kDa was detected as was the appearance of tyrosine phosphorylation on proteins with apparent molecular weights greater than 130 kDa (Figure 1b). Although the profile of proteins showing tyrosine phosphorylation differed slightly depending on the primary antibody used, the main protein clusters with increased phosphotyrosine levels following ET-1 stimulation were consistent (Figure 1a and b). The figure shows a representative immunoblot following 10 min ET-1 (100 nM) stimulation, similar results were obtained at other time points studied.

Stimulation with 100 nM ET-1 induced a rapid, within 20 s, increase in phosphotyrosine content of the 75–85 kDa protein band with an apparent peak in intensity of this band at 2.5 min and which remained elevated above basal levels up to 20 min (Figure 2).

The effect of ET-1 was concentration-dependent. An increase of protein tyrosine phosphorylation of the 75–85 kDa band was detected at 10 nM, approximately 75%, and a greater increase, approximately 175%, at 100 nM following 3 min stimulation (Figure 3). A time point which corresponded to the stable phase of the contractile response to ET-1.

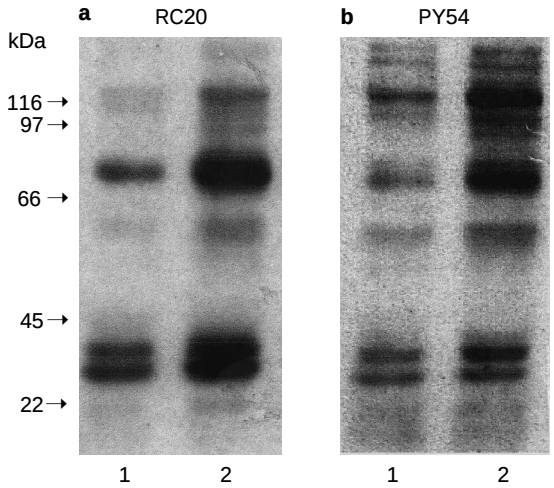


**Figure 1** Endothelin-1 (ET-1)-induced protein tyrosine phosphorylation in rat mesenteric small arteries. Following a 10 min incubation with 100 nM ET-1, vessels were homogenized, run on SDS–PAGE, electrophoretically transferred to membranes and probed with antiphosphotyrosine antibody RC20 (a) as described in Methods. The membrane was stripped and reprobed with antiphosphotyrosine antibody PY54 (b). Lane 1; basal: lane 2; ET-1 100 nM 10 min. The immunoblots shown are representative of at least three experiments.

### *The effect of removal of extracellular calcium on endothelin-1-stimulated protein tyrosine phosphorylation*

The effect of removal of extracellular $Ca^{2+}$ on ET-1-stimulated phosphotyrosine levels was examined during the early (20 s–1 min) and later (5 min) phase of the response. These time points corresponded to the transient contractile response observed with ET-1 in 0 mM $CaCl_2$ and 0.1 mM EGTA solution (see below). Stimulation of mesenteric small arteries with ET-1 (100 nM) in the absence of extracellular $Ca^{2+}$ but in the presence of 0.1 mM or 2 mM EGTA resulted in increases in protein tyrosine phosphorylation of the 75–85 kDa band (Figure 4). In $Ca^{2+}$-free HEPES buffer containing 0.1 mM EGTA the response appeared slightly delayed with no consistent increase in phosphotyrosine levels detected until 1 min of stimulation. The magnitude of the response appeared greater in the presence of 2 mM EGTA compared to 0.1 mM EGTA. However, this was not a consistent observation in all experiments and the increases in relative intensity were significantly different at the 20 s time point only (Figure 4).

### *The effect of tyrphostins A1 and A23 on endothelin-1-stimulated protein tyrosine phosphorylation*

Tyrphostin A1, 100 $\mu$M, had no inhibitory effect on ET-1 stimulated protein tyrosine phosphorylation of the 75–85 kDa band of proteins, during either the early 20 s–1 min or late 10 min phase of the response. Tyrphostin A23, 100 $\mu$M, completely abolished the increase in protein tyrosine phosphorylation in response to ET-1 100 nM (Figure 5).

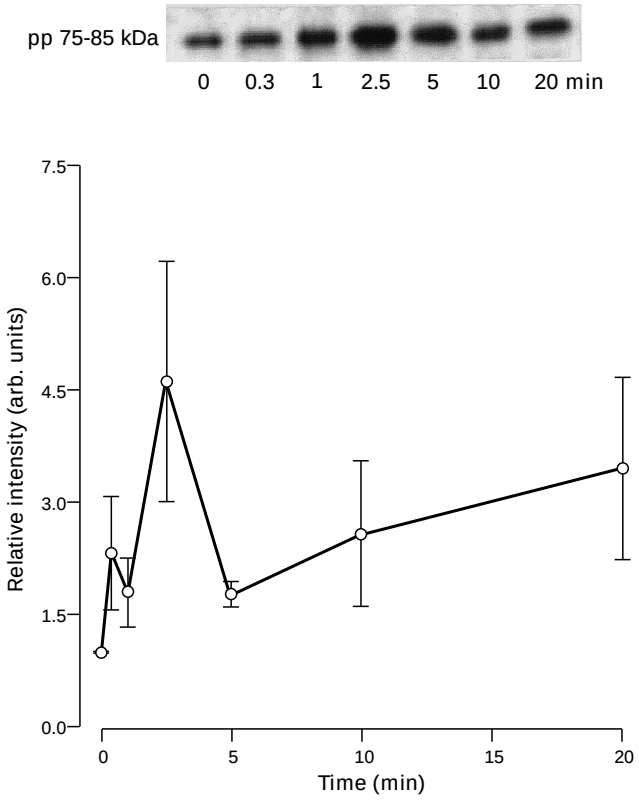


**Figure 2** Time course of endothelin-1 (ET-1)-stimulation of tyrosine phosphorylation of the 75–85 kDa band of proteins. Mesenteric small arteries were stimulated with ET-1 (100 nM) for various times as indicated. The vessels were homogenized, run on SDS–PAGE, electrophorectically transferred to membranes and probed with antiphosphotyrosine antibody RC20 as described in Methods. The autoradiograms were scanned by laser densitometry. The values are relative intensity where $t_0 = 1$ and are the mean of three individual experiments; vertical lines show s.e.mean. A representative immunoblot is shown.

### *Endothelin-1 induced contraction of intact small mesenteric arteries*

Cumulative concentration-response curves demonstrated that ET-1 was an effective vasoconstrictor over the concentration range 1–100 nM (Figure 6a). At a low concentration (1 nM) tension developed gradually reaching a sustained plateau after 5–10 min, at higher concentrations (10 and 100 nM) initial tension development was rapid followed by a sustained tonic contraction (Figure 7a (i)).

### *The effect of tyrphostins A1 and A23 on the contractile response to 100 nM endothelin-1*

In order to investigate the functional significance of the increased protein tyrosine phosphorylation observed following ET-1 stimulation, we studied the effects of tyrphostins A1 and A23 on tension development. The maximal tension developed to ET-1 (100 nM) was reduced by 90% ($P<0.05$) in the presence of 100 $\mu$M tyrphostin A23 but was unchanged by 100 $\mu$M tyrphostin A1 compared to control (Figure 6a). In addition, the profile of the contractile response was altered also in the presence of tyrphostin A23 such that the sustained tonic contractions in response to 10 and 100 nM ET-1 were converted to a transient phasic response (Figure 7a (iii)), although tension did not fall completely to basal levels. Tyrphostin A1 100 $\mu$M did not significantly alter the contractile profile to ET-1 compared to control (Figure 7a (ii)). These data suggest that tyrosine phosphorylation may be involved in the sustained phase of the ET-1-induced contractile responses in small arteries.

### *The effect of tyrphostins A1 and A23 on the contractile response to low concentrations of endothelin-1*

Cumulative concentration-response curves in the presence of 100 $\mu$M tyrphostins A1 and A23 demonstrated that both compounds were equally effective at inhibiting tension developed to low concentrations (1 and 10 nM) of ET-1 (Figure 6a). These data suggested that tyrphostins may have additional effects on the contractile response, by mechanisms not related to tyrosine kinase inhibition. This interpretation is supported by our data demonstrating that A1 did not prevent ET-1-stimulated tyrosine phosphorylation (Figure 5). The effects of tyrphostins on contraction were not restricted solely to receptor-mediated responses as both A1 and A23, at 100 $\mu$M, substantially inhibited $K^+$-induced force in mesenteric arteries (Figure 6b), approximately 64% inhibition of 125 mM KCl-induced tension. Contractions induced by high $K^+$ are due to membrane depolarization and influx of extracellular $Ca^{2+}$, suggesting that tyrphostins may interfere with $Ca^{2+}$ handling in mesenteric small arteries. In contrast to the effects of tyrphostins on ET-1, the inhibitory effect of tyrphostin A1 alone was not overcome at the highest concentration of $K^+$ (125 mM) studied (Figure 6b).

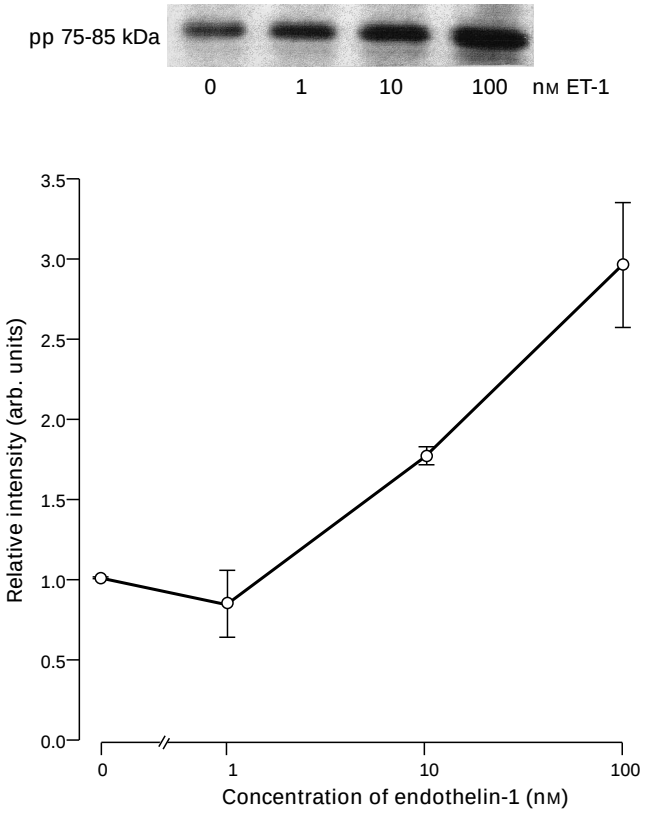


**Figure 3** Concentration relationship of endothelin-1 (ET-1)-stimulation of tyrosine phosphorylation of the 75–85 kDa band of proteins. Mesenteric small arteries were stimulated for 3 min with various concentrations of ET-1 as indicated. The vessels were homogenized, run on SDS–PAGE, electrophoretically transferred to membranes and probed with antiphosphotyrosine antibody RC20 as described in Methods. The autoradiograms were scanned by laser densitometry. The values are relative intensity where basal (ET-1 0 nM) = 1 and are the mean of three individual experiments; vertical lines show s.e.mean. A representative immunoblot is shown.

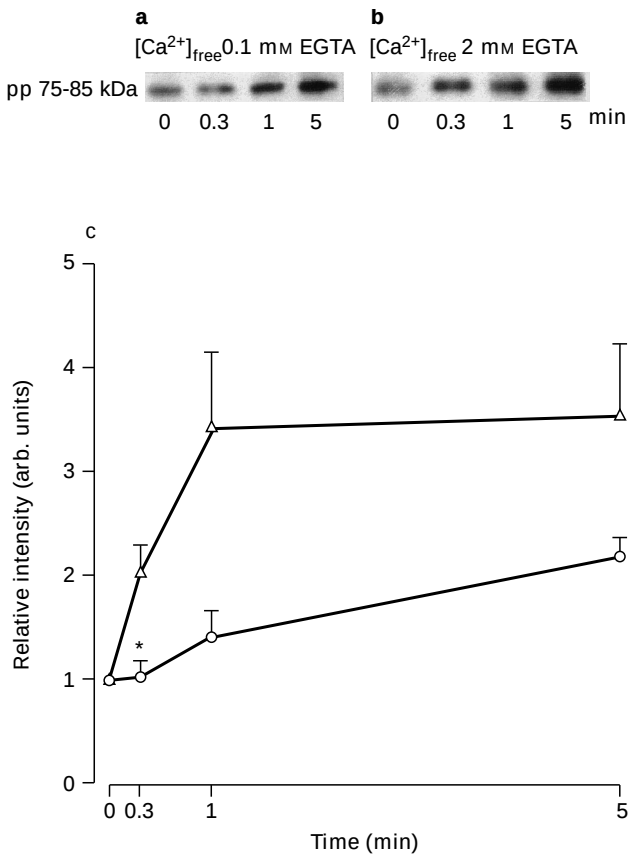


**Figure 4** The effect of removing extracellular calcium on endothelin-1 (ET-1)-induced tyrosine phosphorylation of the 75–85 kDa band of proteins. Mesenteric small arteries were incubated in $Ca^{2+}$-free HEPES buffer containing 0.1 (○) or 2 (△) mM EGTA for 10 min before treatment with ET-1 100 nM for the times indicated. The vessels were homogenized, run on SDS–PAGE, electrophoretically transferred to membranes and probed with antiphosphotyrosine antibody RC20 as described in Methods. The autoradiograms were scanned by laser densitometry. The values are relative intensity where $t_0 = 1$ and are the mean of three individual experiments; vertical lines show s.e.mean. *$P<0.05$ compared to 2 mM EGTA by Student's $t$ test. Representative immunoblots are shown; (b) $Ca^{2+}$-free + 2 mM, (a) $Ca^{2+}$-free + 0.1 mM EGTA which correspond to (△) and (○), respectively, in (c).

### *The effect of tyrphostins A1 and A23 on endothelin-1 stimulated contraction in the absence of extracellular calcium*

In order to investigate if tyrosine kinase inhibition affected the initiation of contraction in response to ET-1, small arteries were incubated in $Ca^{2+}$-free PSS containing 0.1 mM EGTA before and during stimulation. Under these conditions ET-1 (100 nM) induced a transient phasic contraction (Figure 7b (iv)). This response was not altered by either 100 $\mu$M tyrphostin A1 or A23 (maximal tension ET-1 100 nM, vehicle (0.1% DMSO) $1.31 \pm 0.23$, tyrphostin A1 $1.14 \pm 0.26$, tyrphostin A23 $1.06 \pm 0.29$ mN mm$^{-1}$) (Figure 7b (v and vi), suggesting that tyrosine phosphorylation was not involved during this phase of the contractile response. In $Ca^{2+}$-free PSS containing 2 mM EGTA no contractile response to ET-1 (100 nM) was obtained, indicating that no significant increase in intracellular $Ca^{2+}$ had occurred.

### *Endothelin-1-induced calcium sensitization in α-toxin permeabilized mesenteric small arteries*

Our data in intact mesenteric small arteries indicate that increased levels of protein tyrosine phosphorylation in response to ET-1 are important in the tonic phase of the contractile response to this peptide. However, the potent effects of tyrphostins on contraction in intact small arteries through a mechanism other than tyrosine kinase inhibition, possibly $Ca^{2+}$ handling, makes it difficult to interpret the data further. Therefore we investigated the role of phosphorylation in ET-1-induced sensitization of the contractile apparatus to calcium, ie. 'calcium sensitization'. This mechanism contributes substantially to the sustained phase of smooth muscle contraction induced by vasoconstrictor hormones such as ET-1 (Nishimura *et al*., 1992; Somlyo & Somlyo, 1994). α-Toxin permeabilised small arteries were used as this preparation allows intracellular calcium levels to be controlled and yet receptors remain coupled to their intracellular signalling pathways (Kitazawa *et al*., 1989). Cumulative concentration-response curves were obtained to ET-1 in the presence of a constant but low concentration of $Ca^{2+}$ (pCa 6.7) and 10 $\mu$M GTP (Figure 8). Tension developed over the concentrations range 1 to 100 nM ET-1. In the presence of 100 $\mu$M tyrphostin A23, the concentration-response curve to ET-1 was markedly depressed, approximately 50% ($P<0.05$) reduction of force developed to 100 nM ET-1 alone. In constrast, tyrphostin A1 (100 $\mu$M) did not affect ET-1-induced calcium sensitisation, except at the lowest effective concentration tested 1 nM ET-1 (Figure 9).

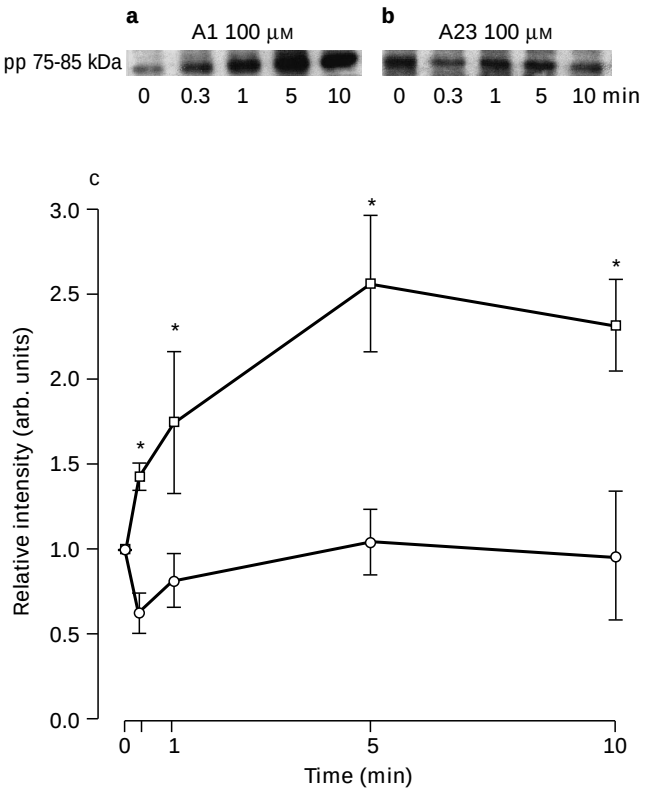


**Figure 5** The effect of tyrphostins A1 (□) and A23 (○) on endothelin-1 (ET-1)-induced tyrosine phosphorylation of the 75–85 kDa band of proteins. Mesenteric small arteries were incubated with ET-1 (100 nM) in the presence of 100 $\mu$M A1 or A23 for various times as indicated. The vessels were homogenized, run on SDS–PAGE, electrophoretically transferred to membranes and probed with antiphosphotyrosine antibody RC20 as described in Methods. The autoradiograms were scanned by laser densitometry. The values are relative intensity where $t_0 = 1$ and are the mean of three individual experiments; vertical lines show s.e.mean. *$P<0.05$ compared to A23 by Student's $t$ test. Representative immunoblots are shown.

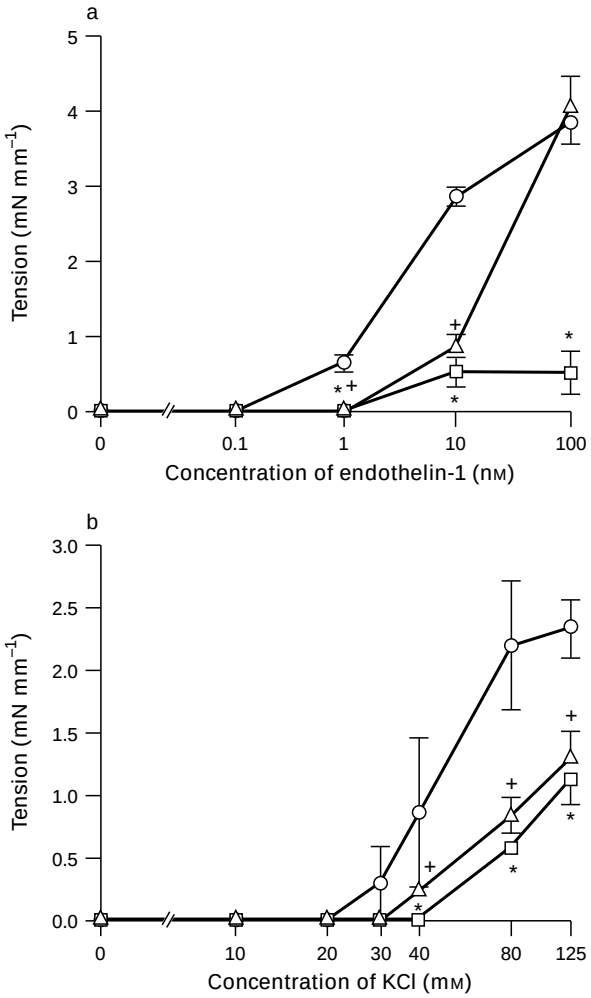


**Figure 6** The effects of tyrphostins A1 and A23 on concentration-tension curves to (a) endothelin-1 (ET-1) and (b) KCl in intact rat mesenteric small arteries. Segments of small artery were mounted in a myograph for the measurement of isometric tension as described in Methods. Cumulative concentration-response curves to (a) ET-1 or (b) KCl were constructed in the presence of: (○) vehicle 0.1% DMSO, (△) 100 $\mu$M A1, (□) 100 $\mu$M A23. Tension was measured when the response had reached a stable plateau. The results are mean for $n=3$; vertical lines show s.e.mean. *$P<0.05$ A23 vs vehicle control, $^{+}P<0.05$ A1 vs vehicle control by ANOVA plus the Least Significant Difference test.

*The effect of tyrphostins A1 and A23 on calcium-induced contraction*

Finally we determined the effects of tyrphostins on $Ca^{2+}$-induced contraction in α-toxin permeabilized small arteries. Vessels were maximally preconstricted with a high concentration of $Ca^{2+}$, pCa 5.0, and 100 μM tyrphostin A1 or A23 added. Tension was reduced 8% and 74% by 100 μM tyrphostin A1 and A23, respectively (mean $n=2$), suggesting that tyrosine phosphorylation may be involved in smooth muscle contraction downstream of $Ca^{2+}$.

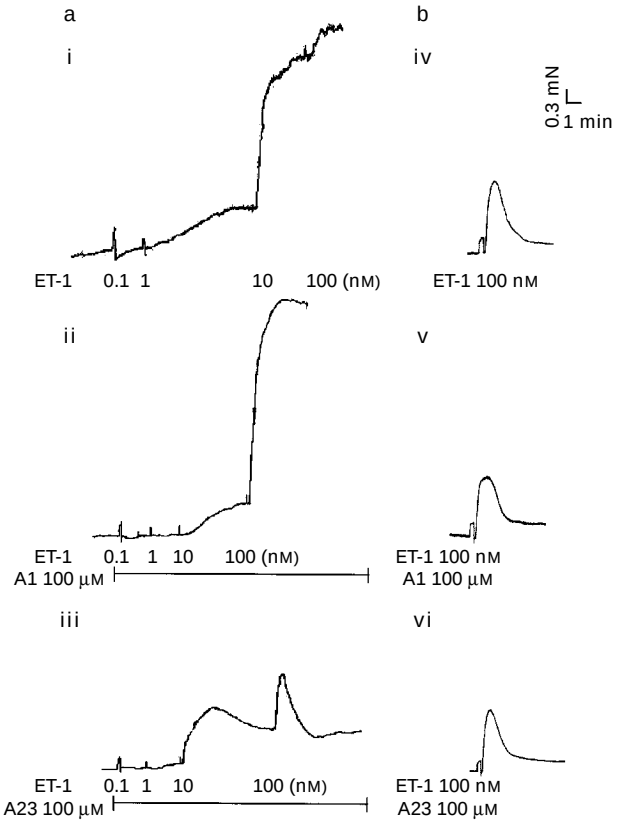


**Figure 7** Contractile response to endothelin-1 (ET-1) in the presence of tyrphostins A1 and A23, and in the absence of extracellular calcium in intact rat mesenteric small arteries. Segments of small artery were mounted in a myograph for the measurement of isometric tension as described in Methods. (a) The tension developed to increasing concentrations of ET-1, 0.1–100 nM (i), in the presence of 100 μM tyrphostin A1 (ii) or tyrphostin A23 (iii) was recorded. (b) Vessels were incubated for 10 min in $Ca^{2+}$-free buffer containing 0.1 mM EGTA, the response to 100 nM ET-1 (iv) in the presence of 100 μM tyrphostin A1 (v) or tyrphostin A23 (vi) was recorded. DMSO (0.1%) vehicle was present during the control ET-1 responses. The tracings are representative of 3 separate experiments.

## Discussion

Given the importance of small artery tone as a determinant of peripheral vascular resistance, we have investigated the regulation of tyrosine phosphorylation following ET-1 stimulation and the role of this pathway in the contractile response. Although it is known that ET-1 is a potent constrictor of vascular smooth muscle, the intracellular signalling cascades

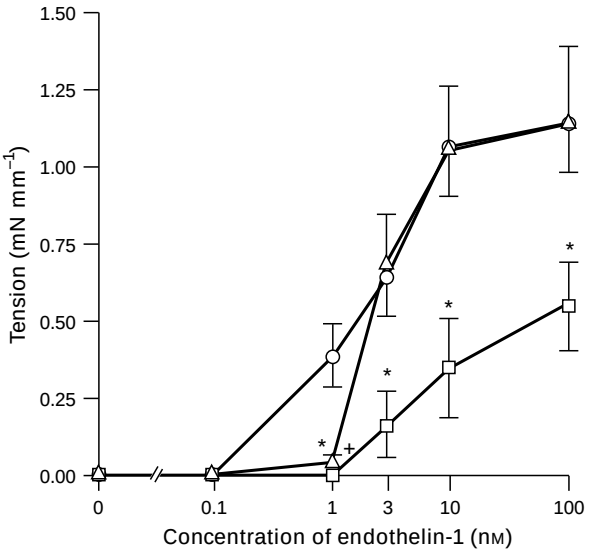


**Figure 9** The effects of tyrphostins A1 and A23 on concentration-tension curves to endothelin-1 (ET-1) in α-toxin permeabilized mesenteric small arteries. Segments of small artery were mounted in a myograph for the measurement of isometric tension. The vessels were permeabilized with *Staphylococcus aureus* α-toxin as described in Methods. Cumulative concentration-response curves to ET-1 were constructed in the presence of: (○) vehicle 0.1% DMSO, (△) 100 μM A1, (□) 100 μM A23. The results are mean for $n=3$; vertical lines show s.e.mean. $^{*}P<0.05$ A23 vs vehicle control, $^{+}P<0.05$ A1 vs vehicle control by ANOVA plus the Least Significant Difference test.

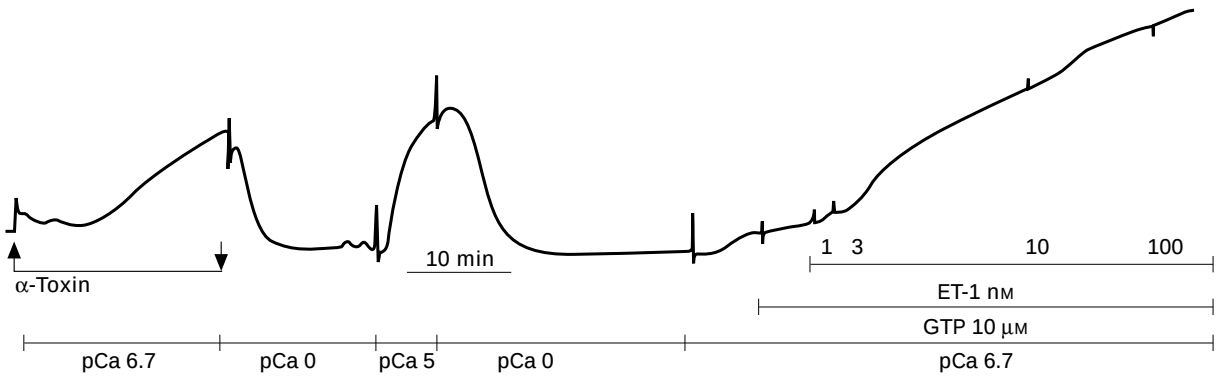


**Figure 8** The contractile responses to calcium and endothelin-1 in α-toxin permeabilized mesenteric small arteries. Segments of small artery were mounted in a myograph for the measurement of isometric tension. The vessels were permeabilized with *Staphylococcus aureus* α-toxin as described in Methods. The contractile response to calcium (pCa 5) was recorded to demonstrate successful permeabilization. Following relaxation in $Ca^{2+}$-free buffer, a cumulative concentration-response curve to ET-1 was constructed in the presence of submaximal calcium (pCa 6.7) and GTP 10 μM. The tracing is representative of three separate experiments.

which bring about this response have not been characterized in intact tissue. In cultured cell systems, ET-1 induced increases of protein tyrosine phosphorylation levels have been linked to the mitogenic effects of the peptide (Koide *et al*., 1992; Zachary *et al*., 1992; Simonson & Herman, 1993). However, evidence from studies with growth factors and $\alpha_1$-adrenoceptor agonists suggests that activation of tyrosine kinases may be involved in contraction (Di Salvo *et al*., 1993b; Hollenberg, 1994; Toma *et al*., 1995), although the mechanisms which regulate tyrosine kinase activity, and the point at which this pathway may be involved in a contractile response, remains unclear. We have carried out both functional and biochemical measurements over a time period relevant to the contractile process: such that precise correlations between intracellular signalling cascades and contraction could be made. Our data demonstrate that ET-1 stimulates tyrosine phosphorylation of a number of proteins in mesenteric small arteries with time and concentration relationships similar to the contractile response. Furthermore, blockade of the increase in phosphorylation with a tyrosine kinase inhibitor reduced the force developed to this agonist. One mechanism by which tyrosine phosphorylation may regulate smooth muscle contraction is through effects on ion channels and calcium influx. The non-receptor membrane-associated tyrosine kinase pp60$^{c\text{-}src}$ increased voltage-operated calcium channel currents in rabbit vascular smooth muscle cells, an effect inhibited by tyrphostin A23 (Wijetunge & Hughes, 1995). Stimulation of pp60$^{c\text{-}src}$ activity by ET-1 occurred in mesangial cells (Simonson & Herman, 1993) and in response to other vasoconstrictor peptides such as angiotensin II in vascular smooth muscle cells (Marrero *et al*., 1995). These data suggest that the src family of tyrosine kinases may be involved in agonist-induced smooth muscle contraction, certainly src activity is decreased in vascular smooth muscle extracts following tyrosine kinase inhibition (Di Salvo *et al*., 1993). Genistein, a tyrosine inhibitor (Akiyama *et al*., 1987), reduced both the tension and intracellular calcium rise in response to noradrenaline in rat mesenteric arteries (Toma *et al*., 1995) suggesting that tyrosine kinases are involved in $Ca^{2+}$ influx in intact preparations. This inhibitor also blocked bradykinin induced $Ca^{2+}$ influx in endothelial cells but did not affect the release of $Ca^{2+}$ from intracellular stores (Fleming *et al*., 1995). In our study tyrosine kinase inhibition altered the profile of the response of intact mesenteric small arteries to high concentrations of ET-1 from a sustained to a transient contraction, suggesting that $Ca^{2+}$ influx may be attenuated. Removal of $Ca^{2+}$ from the extracellular medium also resulted in a transient contraction to ET-1. This part of the response was not affected by tyrosine kinase inhibition, implying that protein tyrosine phosphorylation is not involved in the initial phase of smooth muscle contraction.

Although tyrphostin A1 did not inhibit tyrosine kinase activity, as assessed by protein tyrosine phosphorylation levels, this compound inhibited ET-1 induced contraction, up to 10 nM, in intact arteries. However, unlike tyrphostin A23, the contractile response was sustained in the presence of A1. Furthermore, this effect was not restricted to ET-1 responses as contraction to a nonreceptor stimulus such as high $K^+$ was also attenuated. Recently Toma *et al*. (1995) have also shown that tyrphostin A1 reversibly relaxed noradrenaline- or $K^+$-contracted intact mesenteric arteries with only slightly less potency than tyrphostin A23. In addition, tyrphostin A1 inhibited voltage-operated $Ca^{2+}$ channels in rabbit vascular smooth muscle cells (Wijetunge *et al*., 1992), which could explain the inhibitory effects of this compound on contractions induced by submaximal concentrations of ET-1. In $\alpha$-toxin permeabilised mesenteric arteries, tyrphostin A1 did not inhibit $Ca^{2+}$-induced contraction or ET-1-induced $Ca^{2+}$ sensitisation of force. Taken together these data suggest that the primary site of tyrphostin A1 inhibition of vascular smooth muscle contraction in intact preparations is on plasma membrane-associated ion channels rather than a direct effect on contractility. This action appears to be independent of tyrosine phosphorylation.

Removal of extracellular $Ca^{2+}$ and addition of the chelating agent EGTA did not prevent ET-1-induced protein tyrosine phosphorylation, suggesting that $Ca^{2+}$-independent tyrosine kinases or phosphatases are involved. This is in contrast to studies in mesangial cells which have shown that ET-1 stimulated pp60$^{c\text{-}src}$ activity and tyrosine phosphorylation was dependent on extracellular $Ca^{2+}$ (Simonson *et al*., 1996). Furthermore, agonist-induced protein tyrosine phosphorylation was dependent on either $Ca^{2+}$ influx in vascular smooth muscle (Huckle *et al*., 1992), or a rise in intracellular $Ca^{2+}$ in endothelial cells (Fleming *et al*., 1995). These differences suggest that activation of pathways leading to tyrosine phosphorylation are both cell type- and agonist-specific, further emphasising the importance of this signalling system in vasoconstrictor hormone responses. However, extracellular $Ca^{2+}$ was still required for tonic contraction to ET-1 demonstrating that sustained protein tyrosine phosphorylation was insufficient to maintain tension. Di Salvo *et al*. (1993a) have shown also that force induced by vanadate, a protein tyrosine phosphatase inhibitor, was dependent on extracellular $Ca^{2+}$ even though phosphorylation levels were sustained. Tyrphostins A1 and A23 did not affect the transient contraction induced by ET-1 in the absence of extracellular $Ca^{2+}$, suggesting that tyrosine phosphorylation is not important for the initial events leading to elevation of intracellular $Ca^{2+}$ in vascular smooth muscle. This is in agreement with a study in endothelial cells demonstrating that inhibition of tyrosine kinases specifically attenuated the plateau phase of the $Ca^{2+}$ response, associated with influx of $Ca^{2+}$ rather than the release of $Ca^{2+}$ from intracellular stores, after stimulation with bradykinin (Fleming *et al*., 1995).

Our data suggest that tyrosine phosphorylation may be involved in the sustained phase of ET-1-induced contraction in mesenteric small arteries. A major component of agonist-induced tonic contraction is sensitization of the contractile myofilaments to $Ca^{2+}$ ($Ca^{2+}$ sensitization), such that force is sustained when $Ca^{2+}$ levels decrease (Somlyo & Somlyo, 1994). In rat $\alpha$-toxin permeabilised mesenteric arteries we showed that ET-1 increases force at a constant low $Ca^{2+}$ concentration. The response was dependent on GTP, demonstrating that it was a receptor-mediated effect. Endothelin-1-induced $Ca^{2+}$ sensitisation was concentration-dependent over a similar concentration range as in the intact tissue, and was reduced $\sim$50% by tyrphostin A23 but unchanged by the inactive analogue A1. These data implicate protein tyrosine phosphorylation in $Ca^{2+}$ sensitisation of smooth muscle, especially as ET-1-induced tyrosine phosphorylation is not dependent on extracellular $Ca^{2+}$. The signalling pathways which link receptor activation to enhanced sensitivity of the contractile myofilaments to $Ca^{2+}$ are not fully understood (reviewed Somlyo & Somlyo, 1994). Recently it has been demonstrated that the monomeric G proteins p21Rho and Ras may be involved in $Ca^{2+}$ sensitization induced by GTP$\gamma$S (a non-hydrolyzable analogue of GTP) (Hirata *et al*., 1992; Satoh *et al*., 1993; Noda *et al*., 1995) possibly through inhibition of myosin light chain dephosphorylation (Hirata *et al*., 1992; Noda *et al*., 1995). Tyrphostins partially reversed the $Ca^{2+}$-sensitising effect of GTP-activated Ras in $\beta$-escin permeabilised guinea-pig mesenteric arteries (Satoh *et al*., 1993), implicating tyrosine kinases in this response. However, in rabbit mesenteric arteries inactivation of p21Rho, by ADP-ribosylation, inhibited the $Ca^{2+}$-sensitization induced by carbachol and ET-1 but not phenylephrine (Gong *et al*., 1996), suggesting that the mechanisms coupling receptor activation to smooth muscle contraction are agonist-specific.

Tyrosine kinase inhibition relaxed the $Ca^{2+}$-mediated contraction in $\alpha$-toxin permeabilised mesenteric arteries. This is in contrast to a study in permeabilized guinea-pig mesenteric arteries showing that tyrphostins did not inhibit $Ca^{2+}$-induced contraction (Di Salvo *et al*., 1993b), but in agreement with a more recent study in rat mesenteric small arteries demonstrating inhibition of $Ca^{2+}$-induced contraction by these compounds (Toma *et al*., 1995). It is probable that tyrosine

kinases are involved in $Ca^{2+}$-mediated contraction as there is evidence that $Ca^{2+}$ activates tyrosine kinases in vascular smooth muscle. For instance, the $Ca^{2+}$ ionophore ionomycin, increased protein tyrosine phosphorylation in vascular smooth muscle cells (Tsuda *et al*., 1991). The profile of proteins phosphorylated was similar to that seen following exposure to vasoconstrictor hormones (Tsuda *et al*., 1991). However, ET-1-induced protein tyrosine phosphorylation was independent of extracellular $Ca^{2+}$ suggesting that different/additional kinases or phosphatases were involved. Taken together these data suggest that tyrosine phosphorylation may be involved in both $Ca^{2+}$-mediated contraction and agonst-induced $Ca^{2+}$ sensitisation. In order to determine the precise role of tyrosine phosphorylation in smooth muscle contraction future studies must focus on identifying the substrates phosphorylated and determining whether the primary signal induced by ET-1 is activation of tyrosine kinases or inhibition of tyrosine phosphatases.

In conclusion, we have demonstrated that ET-1 stimulation increases protein tyrosine phosphorylation through a mechanism which is independent of extracellular $Ca^{2+}$ in intact small mesenteric arteries. We have shown that the increased tyrosine phosphorylation coincided temporally with the contractile response to this peptide. Furthermore, inhibition of tyrosine kinase activity and tyrosine phosphorylation attenuated the sustained phase of the contractile response to ET-1 in intact small arteries and inhibited the $Ca^{2+}$ sensitization response in $\alpha$-toxin permeabilised vessels. These data are consistent with a role for the protein tyrosine phosphorylation signalling cascade in the tonic phase of ET-1-induced vascular smooth muscle contraction.

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