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ET_A receptors are the primary mediators of myofilament calcium sensitization induced by ET-1 in rat pulmonary artery smooth muscle: a tyrosine kinase independent pathway

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1 We have investigated the possibility that ET-1 can induce an increase in myofilament calcium sensitivity in pulmonary artery smooth muscle. Arterial rings were permeabilized using α-toxin (120 μg ml^{-1}), in the presence of A23187 (10 μM) to 'knock out' Ca^{2+} stores, and pre-constricted with pCa 6.8 (buffered with 10 mM EGTA). In the presence of this fixed Ca^{2+} concentration, 1 μM ET-1 induced a sustained, reversible constriction of 0.15 mN.

2 Pulmonary arterial rings were freeze-clamped at the peak of the induced constriction (time matched). Subsequent densitometric analysis revealed that ET-1 (1 μM) increased the level of phosphorylated myosin light chains by 34% compared to an 11% increase in the presence of pCa 6.8 alone.

3 In contrast to ET-1, the selective ET_B receptor agonist Sarafotoxin S6C (100 nM) failed to induce a significant constriction.

4 The constriction induced by 1 μM ET-1 was reversibly inhibited when the preparation was pre-incubated (15 min) with the ET_A receptor antagonist BQ 123 (100 μM). The constriction measured 0.13 mN in the absence and 0.07 mN in the presence of 100 μM BQ 123.

5 In contrast, the constriction induced by 1 μM ET-1 measured 0.19 mN in the absence and 0.175 mN following a 15 min pre-incubation with the ET_B antagonist BQ 788 (100 μM).

6 The constriction induced by 1 μM ET-1 measured 0.14 mN in the presence and 0.13 mN following pre-incubation with the tyrosine kinase inhibitor Tyrphostin A23 (100 μM).

7 We conclude that ET-1 induced an increase in myofilament calcium sensitivity in rat pulmonary arteries *via* the activation of ET_A receptors and by a mechanism(s) independent of tyrosine kinase.

Keywords: Smooth muscle; pulmonary artery; ET-1; ET_A; ET_B; calcium sensitization

Abbreviations: ET, endothelin; ET-1, endothelin-1; ET_A, endothelin A receptor; ET_B, endothelin B receptor; HPV, hypoxic pulmonary vasoconstriction; Ca^{2+}, calcium; SX6C, sarafotoxin S6C; MLC, myosin light chains; $[Ca^{2+}]$, calcium concentration; GTP, guanosine 5′ Triphosphate; NP, non-phosphorylated; P1, monophosphorylated; P2, diphosphorylated

Introduction

It is well known that endothelin-1 (ET-1), a member of a family of vasoactive peptides, is one of the most potent constrictors of vascular smooth muscle, and that its effects are mediated by at least two identified receptor subtypes, namely ET_A and ET_B (Yanagisawa *et al*., 1988; Arai *et al*., 1990; Leach *et al*., 1994; Sakurai *et al*., 1990; Sakamoto *et al*., 1991; Yoshida *et al*., 1994; Maguire *et al*., 1996). Recent investigations have suggested that ET-1 may play an important role in the regulation of pulmonary vascular tone and may promote the development of pulmonary hypertension (Chen *et al*., 1997; Bonvallet *et al*., 1994; Prie *et al*., 1997; Langleben *et al*., 1993; Yoshibayashi *et al*., 1991). During pulmonary hypoxia, plasma ET-1 levels are elevated due to increased expression of ET-1 mRNA, and release of ET-1 from pulmonary artery endothelial cells (Giaid *et al*., 1993; Elton *et al*., 1992; Li *et al*., 1994a; 1995), and this is accompanied by an increase in endothelin receptor gene expression in human and rat pulmonary vasculature (Li *et al*., 1994a,b). Furthermore, investigations have shown that orally active ET_A receptor antagonists attenuate hypoxic pulmonary vasoconstriction (HPV) in rats, and suppress the development of hypoxic pulmonary hypertension, right ventricular hypertrophy and pulmonary vascular remodelling (Chen *et al*., 1997; Bonvallet *et al*., 1994). In contrast, the ET_A receptor antagonists had little or no effect on pulmonary arterial pressure in normoxic animals.

When one considers the possible role of ET-1 in mediating HPV, it is of some interest to note that endothelin receptor activation can induce constriction of arterial smooth muscle not only by raising the intracellular Ca^{2+} concentration (Highsmith *et al*., 1992; Pang *et al*., 1989; Kasuya *et al*., 1989; Goto *et al*., 1989; Enoki *et al*., 1995), but by increasing the sensitivity of the contractile apparatus to Ca^{2+} (Nishimura *et al*., 1992; Ohanian *et al*., 1997). The significance of this observation becomes apparent when taken together with the fact that calcium sensitization has also been implicated in the development of HPV (Robertson *et al*., 1995). Hence, pulmonary arteries constrict biphasically when exposed to hypoxia ($\leqslant$50 Torr). An initial transient constriction (phase 1) is followed by a slowly developing and sustained increase in tone (Albarwani *et al*., 1994; Bennie *et al*., 1991; Jin *et al*., 1992; Kovitz *et al*., 1993; Robertson *et al*., 1995), (phase 2). Phase 1 occurs independently of the endothelium and is thought to result from depolarization of the smooth muscle

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cell membrane (Evans *et al*., 1996; Osipenko *et al*., 1997; Yuan *et al*., 1993; Post *et al*., 1992) and Ca^{2+} influx through voltage-gated Ca^{2+} channels (Harder *et al*., 1985; McMurty *et al*., 1976; Madden *et al*., 1992; Cornfield *et al*., 1993; Salvatera & Goldman, 1993; Vadula *et al*., 1993). In contrast, Phase 2 requires the release of a vasoconstrictor from the intact endothelium (Leach *et al*., 1994) and may develop in the presence of a constant cytoplasmic free Ca^{2+} concentration (Robertson *et al*., 1995). It seems possible, therefore, that this endothelially derived vasoconstrictor may be ET-1, and that ET-1 may promote HPV in part by inducing myofilament Ca^{2+} sensitization, which may in turn promote the development of hypoxic pulmonary hypertension.

Until now, however, there have been no investigations of the possibility that ET-1 may induce pulmonary arterial constriction by increasing the sensitivity of the contractile apparatus to calcium. We have, therefore, addressed this question in the present investigation. Our findings suggest that ET_A receptors are the primary mediators of the myofilament calcium sensitization induced by ET-1 in rat pulmonary arteries. The observed calcium sensitization appears to result from an increase in myosin light chain phosphorylation and is mediated by a mechanism(s) independent of the tyrosine kinase pathway which has been proposed to mediate the ET-1 induced increase in myofilament Ca^{2+} sensitivity in systemic arteries (Ohanian *et al*., 1997).

Methods

Permeabilized arterial rings

Male Sprague Dawley rats (313 g) were killed by cervical dislocation. The heart and lungs were removed together and placed in chilled HEPES buffered normal Krebs' solution. In all cases, second order branches off the main intrapulmonary arteries (i.d. 100–400 μm, length 1–2 mm) were dissected out and cleaned of connective tissue using a dissecting microscope. The endothelium of the arteries was then removed by rubbing the inner surface with thin surgical thread (o.d. about 0.1 mm). Vessels were always obtained from the same anatomical position within the lung. Pulmonary artery rings were then tied with thread fibres to a force transducer (AE801, SensoNor a.s., U.K.). The transducer was mounted on a 'bubble plate' (Kitazawa *et al*., 1991) and tension recorded with a flat-bed pen recorder. Pulmonary artery rings were stretched to a length which gave the greatest constriction in 153 mM K^+ solution. After determining the contractile response to K^+ in HEPES buffered Krebs' solution, the strips were incubated in normal relaxing solution (G1) containing 1 mM EGTA. Details of the solutions used have been described previously (Horiuti *et al*., 1986). The calcium concentration of pCa solutions was regulated by the ratio of K_2EGTA and CaEGTA. Strips were permeabilized with 50 μM β-escin or 120 μg ml^{-1} α-toxin (purified from *Staph. Aureus*), respectively, and in the presence of 10 μM A23187 (to deplete intracellular calcium stores), using pCa 6.0 solution at 24°C (Iizuka *et al*., 1994). In all solutions used with β-escin permeabilized strips, 0.1 μM calmodulin was added to compensate for leakage of calmodulin from permeabilized cells.

A maximal calcium response to pCa 4.5 was obtained. After relaxing in G1 solution, the strips were submaximally constricted in a calcium buffer; pCa 6.5–6.8. Once a plateau was reached, ET-1 or Sarafotoxin S6C (SX6C) were added to the bathing solution to study ET receptor mediated calcium sensitization.

Myosin light chain 20 phosphorylation

In order to determine changes in myosin light chain (MLC) phosphorylation in permeabilized pulmonary arterial smooth muscle, rings were rapidly frozen in liquid Freon 22 either at the peak of the ET-1-induced constriction or at the plateau level of a sustained, submaximal Ca^{2+}-induced constriction. Rings were then transferred to frozen acetone containing 10% trichloroacetic acid. The acetone/trichloroacetic acid was exchanged for 100% acetone and rings left to air dry at room temperature. Each sample was pooled from 4 rings in order to provide sufficient protein. Dried rings were homogenized in glycerol sample buffer (containing 1% SDS, 10% glycerol and 20 mM DTT) and subjected to 2-dimensional gel electrophoresis (Nixon *et al*., 1995) consisting of isoelectric focusing (ampholyte range pH 4.5–5.5, Pharmacia Biotech, St. Albans, U.K.) in the first dimension followed by SDS-polyacrylamide gel electrophoresis in the second dimension on 12% polyacrylamide gels. Resolved proteins were transferred onto nitrocellulose (Biorad Labs Ltd., Hemel Hempstead, U.K.) and the membrane stained with colloidal gold solution to reveal protein spots of unphosphorylated, monophosphorylated and diphosphorylated myosin light chains. MLC phosphorylation was quantitatively assessed using a Biorad GS690 densitometer.

Data analysis

The force of constriction is given in mN of tension developed, or normalized to the maximal constriction obtained to pCa 4.5 solution. Data are expressed as the mean $\pm$ s.e.mean unless stated. Data were compared using a Student's *t*-test, unless otherwise indicated, and statistical significance was assumed if $P<0.05$.

Drugs and solutions

All compounds used in this study were obtained from RBI (SIGMA-ALDRICH, U.K.), with the exception of Tyrphostin A23 which was obtained from Semat Technical (U.K.). Endothelin-1, Sarafotoxin S6C, BQ 123 and BQ 788 were dissolved in de-oxygenated H_2O. Tyrphostin A23 was dissolved in DMSO. Stock solutions in DMSO were diluted at least 1 : 1000, after which the vehicle alone had no effect on the response of the preparation to Ca^{2+} or to ET-1.

Results

The effect of endothelin-1 and sarafotoxin S6C in permeabilized pulmonary arterial rings

Either β-escin (50 μM) or α-toxin (120 μg ml^{-1}) permeabilized small pulmonary arterial rings were used to provide a preparation which allowed us to 'clamp' the intracellular calcium concentration ([Ca^{2+}]) at a defined level, while retaining receptor coupled intracellular second messenger pathways (Kitazawa *et al*., 1989; Iizuka *et al*., 1994). Under these conditions a submaximal constriction was first induced by raising the [Ca^{2+}] in the bathing solution from 0 to between pCa 6.8 and pCa 6.5, before the application of ET receptor agonists. Under these 'calcium clamped' conditions the threshold concentration for the ET-1 induced constriction was in the range 30–100 nM (not shown), while 1 μM ET-1 was found to induce a constriction of rat pulmonary arterial rings which measured approximately 50% of the maximum

tension developed in the presence of pCa 4.5 solution. Clearly, with the [Ca^{2+}] clamped and the intracellular stores depleted, the observed constriction could only be due to an increased sensitivity of the contractile apparatus to Ca^{2+}. This concentration of ET-1 was used to provide the standard submaximal response for comparison in the present investigation. It should be noted that although this concentration is at least an order of magnitude greater than that required to induce a maximal constriction in intact (non-permeabilized) pulmonary arteries (Kato *et al*., 1999; see also McCulloch *et al*., 1998), it is to be expected that higher agonist concentrations may be required as thorough permeabilization of smooth muscle preparations, including the larger conduit pulmonary arteries of the rabbit results in a reduction in the efficiency of receptor coupling (Himpens *et al*., 1989). This probably results from the loss/dilution of intracellular messengers and enzymes with time. For this reason, β-escin permeabilized preparations were found to run down dramatically over 30 min even in the presence of added calmodulin (0.1 μM) and GTP (50 μM). We therefore, carried out all subsequent studies using α-toxin permeabilized pulmonary arterial rings.

Figure 1 shows the response of an α-toxin permeabilized pulmonary arterial ring first to an increase in Ca^{2+} from 0 to pCa 6.8 and then to subsequent addition of the selective ET$_B$ receptor agonist SX6C (100 nM) and ET-1 (1 μM), respectively, in the continued presence of pCa 6.8. Addition of pCa 6.8 induced a constriction of 0.07 ± 0.01 mN (24 ± 5% of maximum constriction to pCa 4.5; $n=3$). Subsequent application of the selective ET$_B$ receptor agonist SX6C (100 nM) failed to induce a significant increase in the force of constriction relative to that induced by pCa 6.8 alone. The tension developed by pCa 6.8 alone was 0.07 ± 0.01 mN ($n=3$) and increased to 0.1 ± 0.02 ($n=3$) in the presence of SX6C (an increase of 0.03 ± 0.01 mN; 10 ± 6% of the maximum constriction to pCa 4.5). In marked contrast to the effects of SX6C, a pronounced increase in the sensitivity of the myofilaments to Ca^{2+} was induced by the addition of ET-1 (1 μM), which generated a further 0.15 ± 0.03 mN of tension (49 ± 3% of the maximum constriction to pCa 4.5; $n=3$). Note: In intact rat pulmonary arteries (endothelium denuded), 1 nM SX6C was sufficient to induce a constriction equivalent to that induced by 1 μM ET-1 (Kato *et al*., 1999; see also McCulloch *et al*., 1998).

Effect of ET$_A$ and ET$_B$ receptor antagonists on the response of permeabilized pulmonary arteries to ET-1

In α-toxin permeabilized pulmonary artery rings, application of 1 μM ET-1 increased the constriction induced by pCa 6.8 solution from 0.11 ± 0.02 mN to 0.27 ± 0.02 mN (an increase of 0.15 ± 0.02 mN; $n=9$). Under these conditions the ET-1 (1 μM) induced constriction measured 52 ± 6% of the maximum constriction to pCa 4.5 solution ($n=9$). Figure 2A shows a control response to 1 μM ET-1 in an artery pre-constricted with pCa 6.8 and GTP (50 μM), respectively. Figure 2B shows the constriction induced by 1 μM ET-1 following a 15 min pre-incubation with the selective ET$_A$ receptor antagonist BQ 123 (100 μM; Bonvallet *et al*., 1994). The constriction to 1 μM ET-1 was reduced from 0.13 ± 0.03 mN (53.12 ± 0.05% of the maximum response to pCa 4.5 solution; $n=4$) in the absence to 0.067 ± 0.003 mN (27.92 ± 0.03% of the maximum response to pCa 4.5 solution; $n=4$) in the presence of 100 μM BQ 123 ($P<0.05$). The

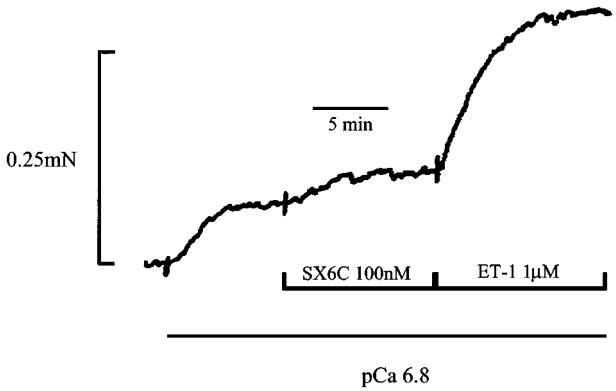

Figure 1 Effect of the selective ET$_B$ receptor agonist SX6C and ET-1 on permeabilized pulmonary artery rings. This figure shows an initial submaximal constriction induced by raising the Ca^{2+} concentration in the bathing solution from 0 to pCa 6.8. In addition the effects of subsequent application of 100 nM SX6C, a selective ET$_B$ receptor agonist, and 1 μM ET-1, respectively, in the continued presence of pCa 6.8 are shown. In this and all subsequent figures pulmonary artery rings were permeabilized using α-toxin (120 μg ml^{-1}), and in the presence of 10 μM A23187 (to deplete intracellular calcium stores), using pCa 6.0 solution at 24°C.

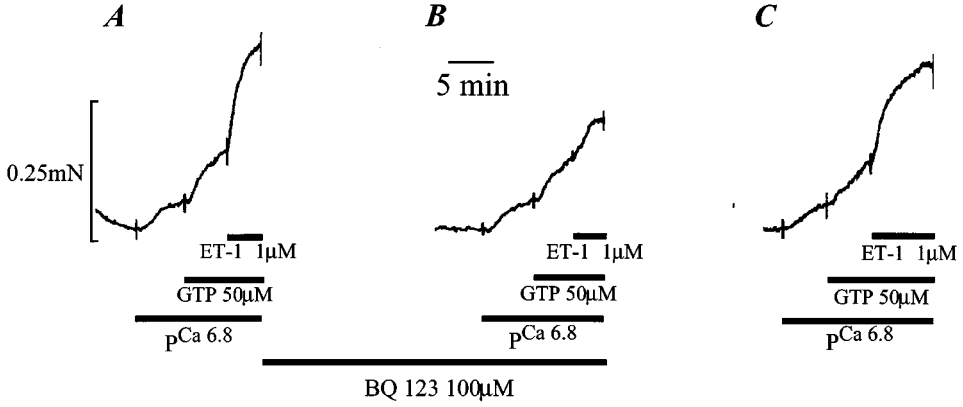

Figure 2 Effect of the ET$_A$ receptor antagonist BQ 123 on the ET-1 induced constriction of permeabilized pulmonary artery rings. (A) shows a typical submaximal constriction to pCa 6.8 and to 50 μM GTP, and the subsequent constriction induced by 1 μM ET-1 in the continued presence of pCa 6.8 and 50 μM GTP. (B) shows the effect of a 15 min pre-incubation with 100 μM BQ 123 on the constriction to pCa 6.8, 50 μM GTP and 1 μM ET-1 respectively. (C) shows the reversal of the effect of 100 μM BQ 123 following a 15 min wash.

constriction to 1 μM ET-1 recovered to 0.12 ± 0.025 mN (43.30 ± 0.01% of the maximum response to pCa 4.5 solution; $n=4$) following a 15 min wash (Figure 2C). In contrast to its effects on the constriction induced by ET-1, 100 μM BQ 123 did not alter the magnitude of the constriction induced by pCa 6.8 solution, which measured 0.32 ± 0.11 mN in the absence and 0.32 ± 0.11 mN in the presence of the ET_A receptor antagonist ($n=4$). Note: The ET-1 induced constriction of rat pulmonary artery rings was unaffected by the presence or absence of applied GTP. We, therefore, excluded GTP from the bathing solution in all other experiments.

Figure 3 shows the response induced by 1 μM ET-1 in an artery pre-constricted with pCa 6.8 (Figure 3A) before and (Figure 3B) after a 15 min incubation with the selective ET_B receptor antagonist BQ 788 (100 μM). In marked contrast to the effects of the selective ET_A receptor antagonist BQ 123 (Figure 2), BQ 788 (100 μM) had no significant effect on the constriction induced by 1 μM ET-1, the tension developed being 0.19 ± 0.05 mN in the absence and 0.175 ± 0.031 mN in the presence of the ET_B receptor antagonist ($n=3$), respectively. A small reduction in the ET-1-induced constriction was observed in the presence of BQ 788 in two out of three preparations. However, a further decline was observed on washing (not shown), which suggests that this was probably due to rundown rather than to the presence of the antagonist *per se*.

Effect of ET-1 on myosin light chain phosphorylation

Figure 4A shows the increase in myosin light chain phosphorylation following the incubation of α-toxin permeabilized pulmonary artery rings with pCa 6.8 solution for 7 min (time-matched to ET-1 treated samples). Three different myosin spots could be distinguished by this method; non-phosphorylated (NP), monophosphorylated (P1) and diphosphorylated (P2) myosin light chains. The diphosphorylated spot appears denser than the monophosphorylated spot due to the presence of unphosphorylated non-muscle myosin light chains which run at the same position as diphosphorylated muscle myosin light chains (Mougios & Barany, 1986). Previous studies of arterial smooth muscle have shown non-muscle myosin light chains to account for 6% of the total myosin (Kitazawa *et al*., 1991) and this has therefore been subtracted from the phosphorylated myosin calculations. The mean densitometric analysis of myosin light chain phosphorylation in pCa 6.8 was 11% ($n=2$ samples, $n=4$ preparations per sample). This is not the peak phosphorylation produced by pCa 6.8 as permeabilized tissues were exposed to Ca^{2+} buffer for 7 min (time-matched experiment) and phosphorylation has been shown to decrease quickly with prolonged exposure (Driska *et al*., 1981). Figure 4B shows the myosin light chain phosphorylation after exposure to pCa 6.8 and 1 μM ET-1. Clearly, ET-1 induced an increase in the monophosphorylated myosin light chains relative to control (pCa 6.8 alone), as would be expected for agonist-induced calcium sensitization (Kitazawa *et al*., 1991). Strips frozen at the peak of the ET-1 induced constriction produced a total phosphorylation (adjusted for non-muscle myosin) of 34% compared to the 11% observed in the controls ($n=2$ samples, $n=4$ preparations per sample). The Ca^{2+} sensitization induced by ET-1 in permeabilized pulmonary arterial rings results, therefore, from a 23% increase in myosin light chain phosphorylation.

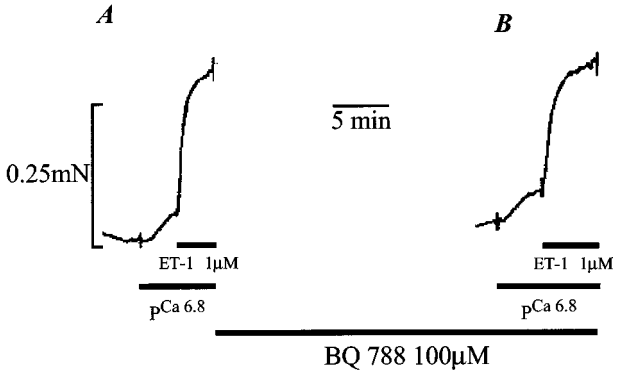

Figure 3 Effect of the ET_B receptor antagonist BQ 788 on the ET-1 induced constriction of permeabilized pulmonary artery rings. (A) shows a submaximal constriction to pCa 6.8, and the subsequent constriction induced by 1 μM ET-1 in the continued presence of pCa 6.8. (B) shows the effect of a 15 min pre-incubation with 100 μM BQ 788 on the constriction to pCa 6.8 and 1 μM ET-1, respectively.

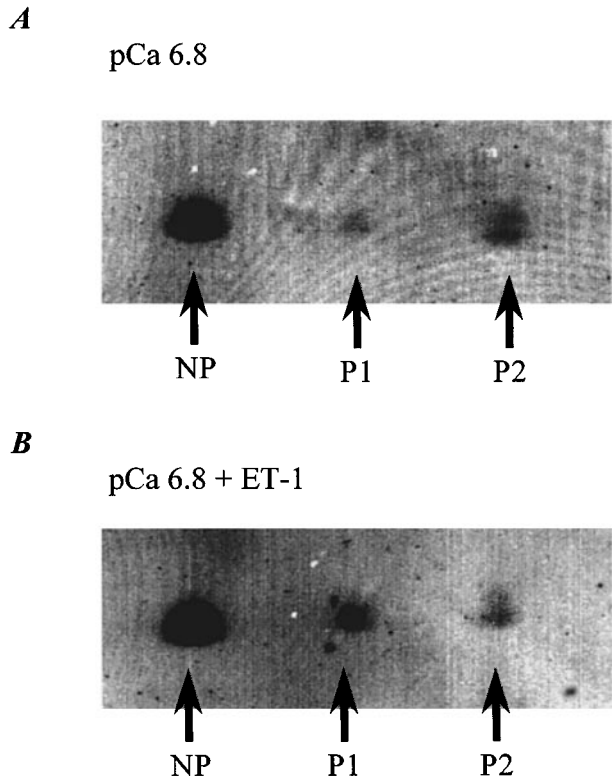

Figure 4 Effect of ET-1 on myofilament phosphorylation in permeabilized pulmonary artery rings. (A) shows the level of unphosphorylated (NP), monophosphorylated (P1) and diphosphorylated (P2) myosin light chains observed in the permeabilized pulmonary artery rings constricted by pCa 6.8 solution alone. (B) shows the level of unphosphorylated (NP), monophosphorylated (P1) and diphosphorylated (P2) myosin light chains observed in the permeabilized pulmonary artery rings constricted by pCa 6.8 solution and 1 μM ET-1. Both (A) and (B) represent the results from four permeabilized artery rings, which were rapidly frozen in liquid Freon 22 at the peak of the induced constriction (time matched at 7 min). The rings were dried and homogenized in glycerol sample buffer and subjected to two-dimensional gel electrophoresis. Resolved proteins were transferred on to nitrocellulose and the membrane stained with colloidal gold solution to reveal protein spots. The phosphorylation level of myofilament phosphorylation was quantitatively assessed using a Biorad GS690 densitometer.

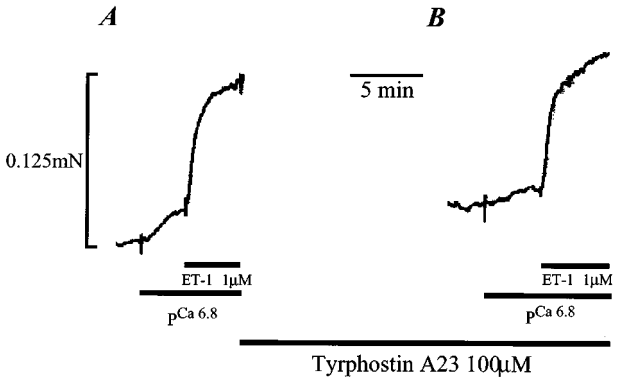

Figure 5 Effect of the tyrosine kinase inhibitor Tyrphostin A23 on the ET-1 induced constriction of permeabilized pulmonary artery rings. (A) shows a submaximal constriction to pCa 6.8, and a subsequent constriction induced by 1 μM ET-1 in the continued presence of pCa 6.8. (B) shows the effect of a 15 min pre-incubation with 100 μM Tyrphostin A23 on the constriction to pCa 6.8 and 1 μM ET-1, respectively.

Effect of the tyrosine kinase inhibitor Tyrphostin A23 on the response of permeabilized pulmonary arteries to ET-1

Figure 5A shows a control response to 1 μM ET-1 in an α-toxin permeabilized pulmonary artery ring which had been pre-constricted by raising the Ca^{2+} concentration to pCa 6.8. Figure 5B shows the constriction induced by 1 μM ET-1 following a 15 min pre-incubation with the tyrosine kinase inhibitor Tyrphostin A23 (100 μM). The constriction to 1 μM ET-1 was unaffected by the presence of 100 μM Tyrphostin A23, the increase in tension measuring 0.143 ± 0.04 mN ($43 \pm 10\%$ of the maximum response to pCa 4.5 solution; $n=4$) in the absence and 0.124 ± 0.06 mN ($37 \pm 12\%$ of the maximum response to pCa 4.5 solution; $n=4$) in the presence of 100 μM Tyrphostin A23. Although not significant, 100 μM Tyrphostin A23 was found to inhibit the pre-constriction to pCa 6.8 solution in three out of four preparations. The increase in tension induced by pCa 6.8 solution was 0.1 ± 0.03 mN ($30 \pm 8\%$ of the maximum response to pCa 4.5 solution; $n=4$) in the absence and 0.069 ± 0.03 mN ($23 \pm 9\%$ of the maximum response to pCa 4.5 solution; $n=4$) in the presence of 100 μM Tyrphostin A23. In addition, exposure of pulmonary arterial rings to 100 μM Tyrphostin A23 resulted in an increase in the resting tone in Ca^{2+} free solution to $20 \pm 4\%$ of the maximum response to pCa 4.5 solution ($n=4$), which may in some way be related to the observed inhibition of the Ca^{2+}-induced constriction.

Discussion

ET-1 increases myofilament calcium sensitivity in pulmonary artery rings by increasing myosin light chain 20 phosphorylation

We have investigated the possibility that ET-1 may induce a constriction of rat pulmonary arteries, at least in part, by increasing the sensitivity of the contractile apparatus to Ca^{2+}. ET-1 was found to induce a sustained and reversible constriction in both β-escin and α-toxin permeabilized pulmonary artery rings, respectively, when the intracellular Ca^{2+} concentration was 'clamped' at a defined level. Importantly, these preparations retain receptor coupled intracellular second messenger pathways, even though the intracellular Ca^{2+} concentration is not allowed to vary in response to applied agonists (Kitazawa *et al*., 1989; Iizuka *et al*., 1994). The constriction induced by ET-1 under these conditions must, therefore, result from an increase in the sensitivity of the contractile apparatus to Ca^{2+}. Moreover, we have shown that the observed calcium sensitization likely results from a concomitant increase in phosphorylated MLC, as would be expected given the findings of previous investigations (e.g. Kitazawa *et al*., 1989). The concentration of ET-1 used to induce a significant increase in myofilament calcium sensitivity was 1–3 orders of magnitude greater than those concentrations required to produce near maximal constriction of intact (i.e. non-permeabilized) rat pulmonary resistance arteries by ourselves and others (Kato *et al*., 1999; McCulloch *et al*., 1998). However, this is not unexpected as thorough permeabilization of smooth muscle preparations, including the larger conduit pulmonary arteries of the rabbit, results in a reduction in the efficiency of receptor coupling *via* a variety of receptor subtypes which probably results from the loss/dilution of intracellular messengers and enzymes with time (Himpens *et al*., 1989). Such a reduction in agonist potency is not, however, observed in poorly/partially permeabilized preparations, but such preparations do not offer a reliable 'calcium clamp'.

The fact that ET-1 is capable of inducing an increase in myofilament calcium sensitivity may be of some significance when taken together with the fact that a vasoconstrictor-induced increase in myofilament Ca^{2+} sensitivity has been implicated in the development of hypoxic pulmonary vasoconstriction (HPV). As mentioned previously, the second phase of acute HPV requires the release of a vasoconstrictor from the intact endothelium (Leach *et al*., 1994) and appears to develop in the presence of a constant cytoplasmic free Ca^{2+} concentration (Robertson *et al*., 1995). Moreover, recent investigations have suggested that ET-1 may play an important role in the regulation of pulmonary vascular tone and may promote the development of hypoxic pulmonary hypertension (Chen *et al*., 1997; Bonvallet *et al*., 1994; Prie *et al*., 1997; Langleben *et al*., 1993; Yoshibayashi *et al*., 1991). Our findings suggest, therefore, that ET-1, or a related peptide, may be responsible for the increase in myofilament Ca^{2+} sensitivity associated with hypoxic pulmonary hypertension. Indirect support for this conclusion comes from the finding that pulmonary hypoxia raises plasma ET-1 levels by increasing the expression of ET-1 mRNA, and increases the release of ET-1 from pulmonary artery endothelial cells (Giaid *et al*., 1993; Elton *et al*., 1992; Li *et al*., 1994a; 1995). However, it should be noted that the suggestion that ET-1 acts as a mediator of HPV remains controversial and a number of authors have shown that ET receptor antagonists are unable to inhibit HPV and it has been proposed that an unidentified vasoconstrictor distinct from ET-1 may act as the primary mediator of HPV (Wadsworth, 1994; Douglas *et al*., 1993; Gaine *et al*., 1998; Ward & Robertson, 1995). Thus, it may be more likely that ET-1 contributes to the development of chronic hypoxic pulmonary hypertension rather than to the acute response to hypoxia (Chen *et al*., 1997; Bonvallet *et al*., 1994; Prie *et al*., 1997; Barton *et al*., 1998).

ET$_A$ but not ET$_B$ receptors mediate the increase in myofilament calcium sensitivity induced by ET-1

In contrast to the effects of ET-1, the selective ET_B receptor agonist SX6C was found to induce little or no constriction

when applied at high concentrations (100 nM) to permeabilized pulmonary artery rings, when the intracellular Ca^{2+} concentration was 'clamped' at a defined level. This finding suggests that ET_B receptor activation may not be directly coupled to those second messenger pathways which promote an increase in myofilament Ca^{2+} sensitivity. Further support for this conclusion was obtained from the finding that high concentrations (100 μM) of the selective ET_B receptor antagonist BQ 788 failed to inhibit the increase in myofilament calcium sensitivity induced by ET-1. However, quite different findings were obtained with the selective ET_A receptor antagonist BQ 123. Pre-incubation of the α-toxin permeabilized pulmonary artery rings with BQ 123, again at 100 μM, caused a marked inhibition (approximately 50%) of the ET-1 induced increase in myofilament Ca^{2+} sensitivity. These findings strongly suggest that the ET-1 induced increase in myofilament calcium sensitivity in resistance-sized rat pulmonary arteries is mediated by ET_A receptors, but not by ET_B receptors and that each receptor subtype may regulate discrete cellular events. These data also suggest that while ET_A receptors may induce a constriction by raising the intracellular calcium concentration and also *via* inducing an increase in myofilament calcium sensitivity, ET_B receptor activation may be exclusively coupled to pathways which mediate calcium influx and intracellular calcium release. This finding is not without significance with respect to HPV, as recent investigations have shown that new orally active ET_A receptor antagonists attenuate HPV in rats, and suppress the development of hypoxic pulmonary hypertension (Chen *et al*., 1997; Bonvallet *et al*., 1994; Prie *et al*., 1997; Barton *et al*., 1998). Thus, we suggest that hypoxic pulmonary hypertension may be due, in part, to the release of ET-1, or a related peptide, from the endothelium in response to hypoxia and that the subsequent activation of ET_A receptors on the smooth muscle cells increases myofilament Ca^{2+} sensitivity, resulting in a sustained vasoconstriction and an increase in pulmonary artery perfusion pressure. Interestingly, previous studies have suggested that ET_A receptors are the primary mediators of the ET-1-induced constriction observed in human pulmonary arteries (Fukuroda *et al*., 1994; Buchan, 1994), although a more recent study has confirmed that vasoconstrictor ET_B receptors are also present in human pulmonary resistance arteries (McCulloch *et al*., 1998).

E-1 induced myofilament Ca^{2+} sensitization may also be involved in post-natal adaption of the pulmonary vasculature, as plasma endothelin levels and ET_A receptor density are both high at birth and exposure to hypoxia from birth prevents their normal reduction and may even increase ETA receptor numbers (Noguchi *et al*., 1997).

The tyrosine kinase pathway does not mediate the calcium sensitization induced by ET-1 in resistance sized rat pulmonary arteries

A recent investigation by Ohanian *et al*. (1997) suggested that ET-1 may induce an increase in myofilament Ca^{2+} sensitivity in mesenteric artery smooth muscle by activating a tyrosine kinase and by subsequent protein tyrosine phosphorylation. We, therefore, investigated the possibility that this pathway mediated the ET-1 induced calcium sensitization observed in rat pulmonary artery smooth muscle. We found that the selective tyrosine kinase inhibitor Tyrphostin A23 failed to inhibit the constriction of α-toxin permeabilized pulmonary artery rings induced by ET-1. In marked contrast, the same concentration of Tyrphostin A23 inhibited the ET-1 induced constriction of rat mesenteric arteries by approximately 50% (Ohanian *et al*., 1997). In agreement with the findings of Ohanian *et al*. (1997) we did, however, find that Tyrphostin A23 inhibited the Ca^{2+}-induced constriction of α-toxin permeabilized pulmonary arteries. These findings suggest that ET-1 may increase myofilament Ca^{2+} sensitivity by a tyrosine kinase independent pathway in rat pulmonary artery smooth muscle, whilst increasing myofilament Ca^{2+} sensitivity through a tyrosine kinase dependent pathway in rat systemic (mesenteric) arteries.

We conclude that the ET-1-induced increase in Ca^{2+} sensitivity in pulmonary artery smooth muscle is primarily mediated by ET_A receptors. Furthermore, our findings suggest that the observed increase in Ca^{2+} sensitivity results from an increase in phosphorylated MLC, which is mediated by a mechanism(s) independent of the tyrosine kinase pathway which has been proposed to mediate ET-1 induced Ca^{2+} sensitization in rat mesenteric arteries. These observations may be of some importance, as the identification of a distinct signal transduction mechanism associated with HPV and the development of pulmonary hypertension could lead to the development of new more effective therapies for this disorder.

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