

POSSIBLE links have been investigated between activation of protein kinase C (PKC) and endothelin (ET) production by small blood vessels. Perfusion pressures were recorded from rat isolated mesenteric artery, with or without the small intestine attached, before and after addition to the perfusate of either ET-1, ET-3 or the PKC activator 12-deoxyphorbol 13-phenylacetate (DOPPA). Rises in perfusion pressure in response to ET-1 (10^{-8} M) or DOPPA (10^{-6} M) were reduced significantly by pre-treatment with either the ET_A receptor antagonist PD151242 (10^{-6} M) or the PKC inhibitor Ro 31-8220 (10^{-6} M). ET-3 (10^{-8} M) had a significant, albeit small, effect only when the gut was still attached to the mesentery. In this latter preparation ET-1 and DOPPA increased the permeability of villi microvessels to colloidal carbon in the perfusate. This effect of DOPPA was reduced by pre-treatment with either PD151242 or Ro 31-8220, but the effects of ET-1 were reduced significantly only by Ro 31-8220. ET-3 (10^{-8} M) was without effect. The results suggest a possible bi-directional link between ET_A receptors and PKC in the intestinal vasculature.

Key words: Colloidal carbon, 12-Deoxyphorbol 13-phenylacetate, Endothelins, Microvascular permeability, PD151242, Protein kinase C, Ro 31-8220, Vasoconstriction

Possible bi-directional link between ET_A receptors and protein kinase C in rat blood vessels

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Introduction

Endothelin (ET) isolated from porcine¹ and from human² endothelial cells (ECs) was shown to consist of three distinct isopeptides, ET-1, ET-2 and ET-3.³ The release of ET-1 from ECs in response to stimulation by angiotensin or vasopressin,⁴ or to shear stress⁵ or to cyclical strain⁶ has been associated with the activation of protein kinase C (PKC). In addition, it has been suggested recently that the release of ETs,⁷ or other vasoconstrictor substances,⁸ may be responsible for the slowly developing vasoconstriction elicited by phorbol esters in rat isolated mesenteric arterioles. The present work was undertaken to seek evidence regarding the possible involvement of ET in this experimental situation, since phorbol esters are well known to activate PKC^{9,10} and one such has been shown to up-regulate the mRNA for preproendothelin-1 in cultured ECs.^{11,12} Phorbol esters have also been shown to increase the permeability to injected colloidal carbon of microvessels in rat small intestinal villi *in vitro*.^{7,13} Thus, the effects of ET-1, ET-3 and 12-deoxyphorbol 13-phenylacetate (DOPPA) on perfusion pressure in rat mesenteric arterioles and on the permeability of microvessels to colloidal carbon in rat villi have been compared. In addition, the effects of pre-treatment with PD151242, an ET_A receptor antagonist,¹⁴ and Ro 31-8220, a specific PKC inhibitor,¹⁵ prior to exposure to ET-1 or DOPPA have been examined.

Methods

The two techniques used in the present experiments have been described in detail elsewhere.^{7,8,16} For both preparations rats weighing 350–500 g were killed by inhalation of chloroform vapour, and the anterior mesenteric artery then cannulated. Subsequently, the mesentery, with or without the small intestine attached to it, was removed to an organ bath containing 50 ml of a physiological salt solution (PSS) at 37°C. The blood vessels were flushed with fresh PSS from a reservoir at 37°C via a roller pump until free of visible blood, and then one or other of the following two procedures was used.

Isolated mesentery: The rate of flow of perfusate was set to give a perfusion pressure of approximately 35 mmHg. The volumes of PSS in the organ bath and in the reservoir were then adjusted to 50 ml in each case, and re-circulation of PSS commenced. When appropriate, PD151242 or Ro 31-8220 was added to the reservoir to give the required concentration. After 10 min of perfusion in this way, the perfusion pressure was noted and then ET-1 or ET-3 or DOPPA was added to the reservoir. Perfusion pressures were recorded at 1 min intervals for the next 15 min. Taking the perfusion pressure at time zero as 0, the rises during the 15 min agonist treatment were calculated. The PSS had the following composition (mM): NaCl 138, KCl 5, NaHCO₃ 10.1, MgCl₂ 1.06, NaH₂PO₄

0.416, CaCl₂, glucose 10 and pH 7.4, and was gassed throughout with 95% O₂ and 5% CO₂.

Mesentery with small intestine attached: The method was similar to that described above except that the rate of flow of perfusate was set at 10 ml/min before re-circulation of perfusate began, and gelatin (2%) was present in the PSS throughout to reduce oedema formation in the gut. After 10 min of re-circulation of the gelatin-containing perfusate (GPSS), with or without added PD151242 or Ro 31-8220, the perfusion pressure was noted and either ET-1 or ET-3 or DOPPA was added. Perfusion pressures at 1 min intervals were recorded for 5 min, after which fresh GPSS was perfused for 1 min, followed by a bolus of 0.5 ml colloidal carbon suspension injected near the cannula.¹⁶ After a further 5 min period of perfusion with GPSS, 2 ml rat washed red blood cells were injected to provide a visual check that vessels in the gut wall were patent. Then the preparation was removed from the organ bath and six pieces of small intestine, each about 4 cm long, were cut, starting from the caecal end. Each was flushed with normal saline, opened lengthways and stapled to a xylene-resistant coverslip. After fixing in formal saline, dehydrating in graded alcohols and clearing in xylene overnight, the specimens were mounted, mucosal surface uppermost, in DPX mountant. Five areas per coverslip were photographed using Ilford FP4 black and white film at $\times 100$ magnification. Negative micrographs were then subjected to image analysis, using a Seescan system (Cambridge, UK), to assess the amount of colloidal carbon trapped in the villi microvessels, as described in detail earlier.¹⁶ A mean value for each piece of small intestine was calculated and used to give a mean value for each animal. Mean values for each treatment group were then compared statistically. The mean rises in perfusion pressure recorded at the end of the 5 min period of ET-1, ET-3 or DOPPA treatment (taking that at time zero as 0) were also compared.

Statistical analysis: Bonferroni's test for comparing more than one treatment group with a single control group was used.

Chemicals used: ET-1 and ET-3 were obtained from Sigma Chemical Co., Poole, UK; DOPPA was obtained from Calbiochem-Novachem, Nottingham, UK; PD151242 was a gift from Dr A. Doherty, Parke Davis, Ann Arbor, MI, USA; Ro 31-8220 was a gift from Dr G. Lawton, Roche Products, Welwyn Garden City, UK; DPX mountant was obtained from BDH, Poole, UK; gelatin was obtained from Thornton Ross, Huddersfield, UK; Thermanox^R xylene-resistant coverslips were obtained from Nunc Inc., Naperville, IL, USA; and colloidal carbon (Gunther Wagner, batch C11/1431a) was obtained from Pelikan Inks, Hanover, Germany. DOPPA and Ro 31-8220 were

dissolved in dimethyl sulphoxide, which, in the concentration used, was biologically inert; ET-1, ET-3 and PD151242 were dissolved in water, the latter with the aid of a little Na₂CO₃. The dose volume used was 0.2 ml.

Results

In the isolated mesentery without small intestine ET-1 (10⁻⁸ M) caused a very marked, but slow rise in perfusion pressure, which reached a peak after 13 min of perfusion (Fig. 1). The response was completely abolished by pre-treatment with PD151242 (10⁻⁶ M) and significantly reduced by pre-treatment with Ro 31-8220 (10⁻⁶ M). DOPPA (10⁻⁶ M) also caused a gradual rise in perfusion pressure, confirming results from earlier work.⁸ This response was significantly reduced by pre-treatment with PD151242, whereas pre-treatment with Ro 31-8220 was shown previously to abolish the effect.⁸ Perfusion of the mesenteric arterioles with ET-1 (10⁻⁹ M) generated a maximum rise of only 6.5 \pm 2.6 mmHg. Perfusion with PSS alone (controls), or ET-3 (10⁻⁸ M), PD151242 alone or Ro 31-8220 alone had no effect on perfusion pressure over a 15 min period.

Perfusion of the vasculature in the isolated mesentery with its small intestine still attached

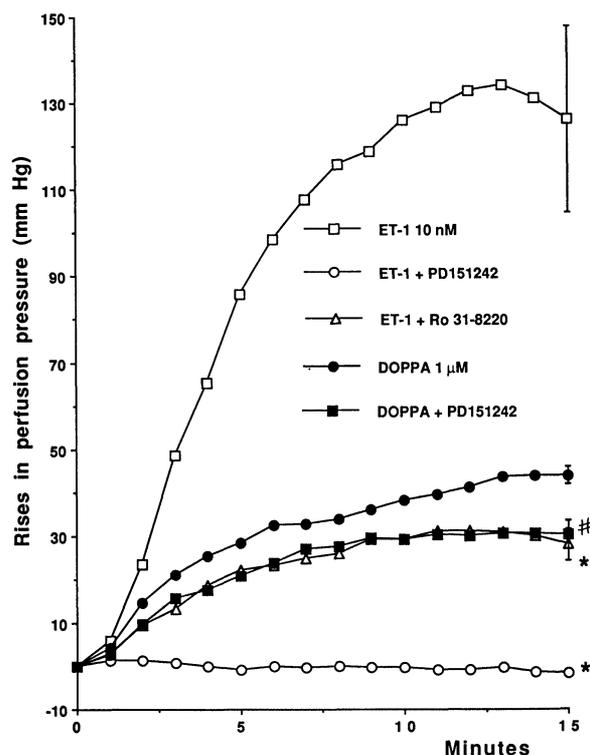


FIG. 1. Changes in perfusion pressure in arterioles of rat isolated mesentery during perfusion with ET-1 (10⁻⁸ M) or DOPPA (10⁻⁶ M) in the presence or absence of PD151242 (10⁻⁶ M) or Ro 31-8220 (10⁻⁶ M). *A significant difference exists compared with ET-1 alone, or #compared with DOPPA alone ($p < 0.05$, Bonferroni's test). Data points (\pm S.E.M.) are derived from three to five observations.

yielded results which were somewhat different from those obtained with the mesentery alone. Perfusion with GPSS containing either ET-1 (10⁻⁸M) or DOPPA (10⁻⁶M) resulted in modest but highly significant rises in perfusion pressure (Table 1). ET-3 (10⁻⁸M) also evoked a significant although much smaller pressor effect. ET-1 (10⁻⁹M) was again inactive in this respect (Table 1). Pre-treatment with either PD151242 or Ro 31-8220 significantly reduced the effects of both ET-1 (10⁻⁸M) and DOPPA (10⁻⁶M).

ET-1 (10⁻⁸M) was found to be less active than DOPPA when assessed for its ability to induce colloidal carbon leakage in villi microvessels (Table 2), although both compounds induced effects that were significantly greater than control values. Pre-treatment with Ro 31-8220 significantly reduced the effects of both ET-1 and DOPPA, but PD151242, while tending to reduce the effects of ET-1, only significantly reduced the effects of DOPPA (Table 2). ET-1 (10⁻⁹M) and ET-3 (10⁻⁸M) did not significantly affect the leakage of colloidal carbon (Table 2).

Discussion

The three ET isopeptides that have been described,³ act upon two ET receptors, ET_A and ET_B.¹⁷ ET_A receptors have a higher affinity for ET-1 and ET-2 than for ET-3, whereas ET_B receptors have a similar affinity for all three isopeptides. In arterial blood vessels activation of ET_A receptors is associated with smooth muscle contraction, whereas activation of

ET_B receptors sometimes produces smooth muscle relaxation, the latter possibly via the release of nitric oxide.¹⁸ Coupling of both types of ET receptor to phospholipase C via a G protein has been demonstrated.¹⁹ However, in human and rabbit saphenous veins, stimulation of both ET_A and ET_B receptors is associated with smooth muscle contraction,^{20,21} the ET_A-mediated response being PKC-dependent and the ET_B-mediated response being PKC-independent.²⁰

The present results, using the mesenteric vessels, confirm other reports that ET-1 is a more potent vasoconstrictor than ET-3,^{3, 21-23} and suggest that in isolated mesenteric arterioles the constrictor effect of ET-1 is mediated via ET_A receptors, since it is abolished by pre-treatment with the specific ET_A receptor antagonist PD151242, and significantly reduced by pre-treatment with the specific PKC inhibitor Ro 31-8220. The virtual lack of effect of the same concentration of ET-3 in this situation also suggests the absence of functionally important ET_B receptors in mesenteric arterioles. Evidence from other workers indicates that ET_A receptors are located mainly on the arterial side of the circulation and ET_B receptors on the venous side,²⁴ although there are variations between species and organs.^{23,24}

When the small intestine remains attached to the mesentery, capillaries and venules, as well as arterioles, are involved in the perfusion. In the light of work cited above²⁴ this would provide an opportunity for stimulation of ET_B receptors, which might

Table 1. Effects of ET-1, ET-3 and DOPPA on perfusion pressures in rat isolated mesenteric and small intestinal vessels in the presence and absence of PD151242 (10⁻⁶ M) or Ro 31-8220 (10⁻⁶ M)

Treatment	Alterations in perfusion pressure (mmHg) after 5 min perfusion ^{ab}						
	Concentration (M)	<i>n</i>	GPSS	<i>n</i>	GPSS + PD151242	<i>n</i>	GPSS + Ro 31-8220
Control	—	10	-2.70 ± 0.90	3	-2.33 ± 0.33	4	-0.5 ± 1.19
ET-1	10 ⁻⁸	7	32.71 ± 2.50 ^c	4	6.75 ± 3.33 ^d	4	3.75 ± 0.75 ^d
ET-1	10 ⁻⁹	4	0.25 ± 0.85	—	—	—	—
ET-3	10 ⁻⁸	5	2.80 ± 0.58 ^c	—	—	—	—
DOPPA	10 ⁻⁶	8	36.75 ± 7.34 ^c	5	14.80 ± 2.96 ^d	5	13.2 ± 2.69 ^{d,e}

^aPerfusion pressure at the beginning of the 5 min perfusion period was taken as 0. ^bResults are mean ± S.E.M. ^c*p* < 0.05 (Bonferroni's test) compared with control values. ^d*p* < 0.05 (Bonferroni's test) compared with agonist alone. ^eValue taken from Reference 8.

Table 2. Leakage of colloidal carbon in microvessels of villi in the rat small intestine *in vitro* in response to ET-1, ET-3 or DOPPA, and the effects of pre-treatment with PD151242 (10⁻⁶ M) or Ro 31-8220 (10⁻⁶ M)

Treatment	Amount of CC, determined by image analysis, assessed as % of frame area ^{ab}						
	Concentration (M)	<i>n</i>	GPSS	<i>n</i>	GPSS + PD151242	<i>n</i>	GPSS + Ro 31-8220
Control	—	10	1.05 ± 0.16	3	1.20 ± 0.08	4	0.83 ± 0.10
ET-1	10 ⁻⁸	7	2.05 ± 0.28 ^c	4	1.39 ± 0.38	4	0.80 ± 0.11 ^d
ET-1	10 ⁻⁹	4	1.62 ± 0.49	—	—	—	—
ET-3	10 ⁻⁸	5	0.65 ± 0.11	—	—	—	—
DOPPA	10 ⁻⁶	8	3.74 ± 0.54 ^c	5	2.18 ± 0.24 ^d	5	0.90 ± 0.12 ^{d,e}

^aEach frame represents one negative micrograph magnified approximately 20 times. ^bResults are given as mean ± S.E.M. ^c*p* < 0.05 (Bonferroni's test) compared with control value. ^d*p* < 0.05 (Bonferroni's test) compared with agonist alone. ^eValue taken from Reference 8.

then have a relaxant effect¹⁸ in addition to the contracting effect brought about via arterial ET_A receptors. Activation of two such opposing effects might explain the smaller rise in perfusion pressure brought about by ET-1 (32.75 ± 2.50 mmHg after 5 min of perfusion in the mesentery plus small intestine, compared with 85.80 ± 11.60 mmHg after 5 min perfusion in the mesentery alone). Alternatively, or in addition, differences in perfusion pressure rises in the two preparations may reflect differences in the flow rates of perfusate used.

PKC-dependent actions of ETs in native vascular smooth muscle^{20,25,26} and in cultured vascular smooth muscle cells^{27,28} have been demonstrated, as has a release of ETs by activation of PKC by phorbol esters.^{11,12} Therefore, since the effects of DOPPA were reduced in both preparations used in the present work, by pre-treatment with either the ET_A receptor antagonist or the PKC inhibitor, the possibility of a bi-directional link between these two processes arises. On the basis of present evidence, however, it is not possible to distinguish between mediation by ET-1 or ET-2, or by a mixture of ET-1 and ET-2, in these situations.

A similar link may also be involved in the DOPPA- and the ET-1 induced increases in vascular permeability that were manifest as a leakage of colloidal carbon into microvessel walls in small intestinal villi (Table 2). However, while ET-3 (10⁻⁸ M) did not increase colloidal carbon leakage in the present experiments, other workers have shown that an application of ET-3 (10⁻¹⁰ M) in conjunction with an ET_A receptor antagonist increased microvascular permeability to FITC-labelled albumin in rat mesentery.²⁹ *In vivo*, the ET-1-induced increase in vascular permeability to Evans Blue dye appeared to be mediated via release of platelet-activating factor³⁰ but we have no information at the moment as to whether this also operated in our experimental situation. We have, in fact, shown that DOPPA-induced colloidal carbon leakage was enhanced by pre-treatment with indomethacin¹³ suggesting a possible inhibitory effect of prostanoids.

Increases in microvascular permeability in response to various inflammatory agonists have been attributed to an increase in cytoplasmic Ca²⁺ levels in Ecs, either as a result of influx from the extracellular medium or by release from intracellular stores.^{31,32} Such increases in Ca²⁺ levels have been demonstrated in response to bradykinin,^{33,34} to platelet activating factor³⁵ and, more recently, to ET-1,^{36,37} and are thought to lead to EC contraction. This results from an actomyosin shortening, brought about by phosphorylation of myosin light chains via myosin light chain kinase, which is a Ca²⁺-calmodulin-dependent process.³⁸ Myosin light chains can also be phosphorylated by PKC,³⁹ as can the thin-filament regulatory protein caldesmon.⁴⁰ Therefore, previous

reports showing that stimulation of ET-1 receptors induces a phosphorylation of myosin light chains and of caldesmon in arterial smooth muscle^{41,42} are important in the present context, and support the suggested bi-directional link between activation of ET_A receptors and activation of PKC.

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Received 7 September 1994;
accepted in revised form 14 October 1994



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