Molecular biology and biochemistry of the endothelins

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Endothelin-1 (ET-1), the first member of the newly discovered mammalian endothelin family of biologically active peptides, was originally identified as a 21 residue potent vasoconstrictor peptide in vascular endothelial cells. However, it has since been demonstrated to possess a wide variety of pharmacological activities in tissues both within and outside the cardiovascular system, and peptides with a striking similarity to ET-1 have been found to be the major toxic component of a snake venom. Moreover, recent studies have suggested that mammals including humans produce three distinct members of this peptide family, ET-1, ET-2 and ET-3, which may have different profiles of biological activity, and may act on distinct subtypes of endothelin receptor. Masashi Yanagisawa and Tomoh Masaki *review the current status of the biochemistry and molecular biology of endothelin.*

The discovery that the vascular endothelium releases a variety of vasoactive substances – including prostacyclin and endothelium-derived relaxing factor (now identified as nitric oxide) – led to the realization that it plays a critical role in the regulation of vascular smooth muscle tone. The picture has been complicated by the observation that the endothelium also releases contracting factor(s) in response to anoxia, the calcium ionophore calcimycin (A23187) and various other stimuli [see Vanhoutte, P. M. and Katusic, Z. S. (1988) *TiPS* 9, 229–230]. An endothelium-derived vasoconstrictor peptide, termed endothelin, has now been identified; remarkably, its gene was cloned and sequenced before the peptide had been pharmacologically characterized [Ref. 1, and see Hiley, C. R. (1989) *TiPS* 10, 47–49].

Endothelin-1 (ET-1) was originally identified as a 21 amino acid, potent vasoconstrictor peptide in the culture supernatant of porcine aortic endothelial cells, but has subsequently been found to exert a wide variety of effects on both vascular and non-vascular tissues[2]. It is synthesized as a precursor peptide of 203 amino acids, containing a signal sequence, which is proteolytically cleaved to produce the 38 (human) or 39 (porcine) amino acid intermediate big endothelin-1 (big ET-1). This is subsequently processed to mature ET-1 (Refs 1 and 2) by a putative 'endothelin-converting enzyme'.

ET-1 provokes strong and sustained constriction in isolated vascular smooth muscle preparations, in almost all animal species and vascular regions examined, including microvessels[1,3]. When injected i.v. it produces an extremely long-lasting pressor response; chronic infusion with low doses of ET-1 (3–5 pmol kg^{-1} min^{-1}) for seven days produces sustained elevation of blood pressure in conscious rats[4]. Other vascular effects of ET-1 include the stimulation of release of eicosanoids and endothelium-derived relaxing factor (EDRF) from perfused vascular beds[5]. Pretreatment with indometacin augments the ET-1-induced pressor response in rats; this may, at least in part, be a result of an inhibition of formation of the vasodepressor eicosanoids. In perfused, precontracted rat mesenteries, infusion of ET-1 produces a transient depressor response which is inhibited by oxyhemoglobin, suggesting that the response is mediated by ET-1-induced EDRF release[5]. An i.v. bolus of ET-1 also causes a transient decrease of systemic arterial pressure accompanied by regional vasodilator responses in anesthetized rats and cats[6,7]. However, it remains to be established conclusively whether these vasodepressor actions *in vivo* are due to the release of EDRF in response to ET-1.

ET-1 also has a wide spectrum of pharmacological effects in tissues other than blood vessels (Table I). In agreement with these

TABLE I. Role of endothelin in non-vascular tissues

Effect	Ref.
• Constriction of airway, intestinal and uterine smooth muscle	Lagente, V. *et al.* (1989) *Biochem. Biophys. Res. Commun.* 158, 625–632; de Nucci, G. *et al.* (1988) *Proc. Natl Acad. Sci. USA* 85, 9797–9800; Kozuka, M. *et al.* (1989) *Biochem. Biophys. Res. Commun.* 159, 317–323
• Cardiac effects, including positive inotropic and chronotropic actions and stimulation of atrial natriuretic peptide release	Ishikawa, T. *et al.* (1988) *Am. J. Physiol.* 255, H970–H973; Fukuda, Y. *et al.* (1988) *Biochem. Biophys. Res. Commun.* 155, 167–172
• Non-vascular renal effects, including inhibition of renin release from isolated glomeruli, inhibition of ouabain-sensitive Na^+/K^+-transporting ATPase in inner medullary collecting duct cells and blockade of the antidiuretic effect of vasopressin *in vivo*	Rakugi, H. *et al.* (1988) *Biochem. Biophys. Res. Commun.* 155, 1244–1247; Zeidel, M. L. *et al.* (1989) *J. Vasc. Med. Biol.* 1, 126; Goetz, K. *et al. Proc. Soc. Exp. Biol. Med.* (in press)
• Mitogenic actions in cultured vascular smooth muscle cells, mesengial cells and Swiss 3T3 fibroblasts	Komuro, I. *et al.* (1988) *FEBS Lett.* 238, 249–252; Simonson, M. S. *et al.* (1989) *J. Clin. Invest.* 83, 708–712; Takuwa, N. *et al.* (1989) *J. Biol. Chem.* 264, 7856–7861
• Modulation of catecholamine release from sympathetic termini and adrenomedullary chromaffin cells	Tabuchi, Y. *et al.* (1989) *Biochem. Biophys. Res. Commun.* 161, 803–808; Boarder, M. R. (1989) *J. Cardiovasc. Pharmacol.* 13 (Suppl.), S223–S224
• Stimulation of aldosterone release in adrenocortical glomerulosa cells	Cozza, E. N. *et al. J. Clin. Invest.* (in press)
• Effects on the CNS, including potent pressor action (when injected i.c.v.) mediated by increased sympathetic outflow and stimulation of substance P release from spinal cord of rat	Takahashi, H. pers. commun.; Yoshizawa, T. *et al. Neurosci. Lett.* (in press)

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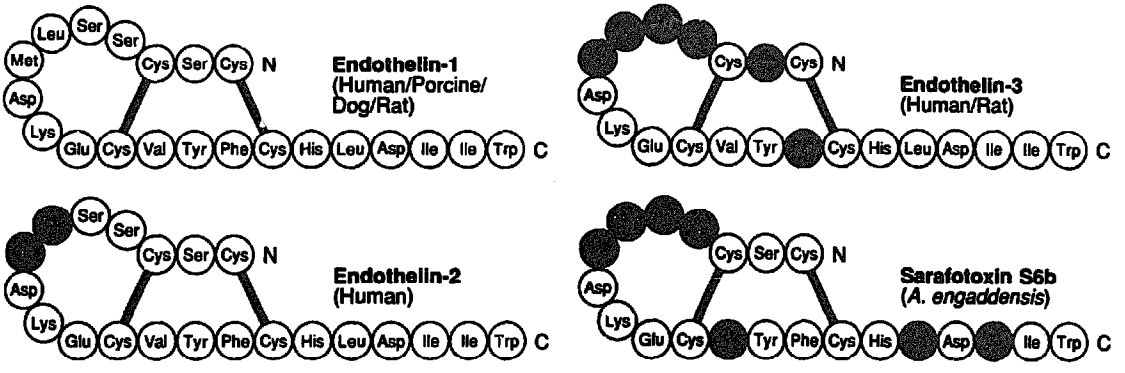


*Fig. 1. Amino acid sequences of peptides from the endothelin family[2,12,13,41]. **Filled circles**, amino acid residues different from those in ET-1 sequence. Since ET-2 and ET-3 have not yet been fully identified at the peptide level, the disulfide-bond topologies and the chain lengths of these predicted peptides are drawn by analogy with ET-1.*

observations, tissue autoradiographic studies with ^{125}I-labeled ET-1 in the rat demonstrate the localization of specific high affinity binding sites for ET-1, not only within the vascular system but also in other tissues including the intestine, heart, lungs, kidneys (most prominently in the glomeruli and papillar regions), adrenal glands (especially medulla) and the brain[8–10].

The mammalian endothelin family

A group of peptide toxins from the venom of burrowing asp *Atractaspis engaddensis*, the sarafotoxins S6, which cause severe coronary vasospasm in snake-bite victims, exhibit a remarkable structural and functional homology to ET-1 [see Kloog, Y. and Sokolovsky, M. (1989) *TiPS* 10, 212–214]; the similarity is so extensive that these mammalian and snake peptides almost certainly have a common evolutionary origin (Fig. 1). As anticipated, sarafotoxins and ET-1 bind competitively to the same specific receptor sites in rat cardiac membrane[11].

The presence of functional cognate peptides of ET-1 in the venomous glands of the snake, an organ apparently unrelated to the vascular endothelium, together with the observations that ET-1 has specific binding sites and pharmacological activities in a wide variety of tissues, suggested that ET-1 might not be the only mammalian version of this family of peptides, and that ET-1 might be produced in tissues other than the vascular endothelium. Indeed, genomic Southern blot analysis under a low hybridization stringency with a short synthetic DNA probe that encodes ET-1 reveals that three genes related to ET-1 are present in the human, porcine and rat genomes[12].

Cloning and sequence analysis of the three genes in humans shows that one of these genes encodes ET-1. The other two genes encode ET-2 and ET-3, which are similar to ET-1 but differ in two and six amino acid positions, respectively (Fig. 1). The sequence of human ET-3 is identical to a sequence previously found in the rat genome[13], which is therefore now considered to be the rat ET-3 gene. It is currently believed that many mammalian species produce three isopeptides from the endothelin family; these are probably identical with or very similar to human ET-1, ET-2 and ET-3. Furthermore, ET-2 and ET-3 may, like ET-1, be processed from larger intermediates, big ET-2 and big ET-3, since the deduced amino acid sequences of the three genes are nearly identical around the Trp21 residues at which the cleavage occurs[12].

It appears that vascular endothelial cells do not produce ET-2 or ET-3; only ET-1 can be detected in endothelial cells (or in the culture supernatant) either at the peptide level or at the mRNA level. The expression of ET-2 has not yet been convincingly demonstrated in any tissues. A recent study has demonstrated that ET-3-like immunoreactive material is present in a porcine brain homogenate. Furthermore, purification and sequence determination of this material have shown that it is co-eluted with synthetic human/rat ET-3 on a reverse-phase HPLC and that its partial amino acid sequence is identical to human/rat ET-3 (O. Shinmi *et al.*, unpublished). Thus, ET-3 may be a neural form of endothelin.

The question of whether ET-1 is expressed in non-endothelial cells has not been completely resolved, although ET-1 like immunoreactivity has recently been detected in certain types of neuron including motor neurons and primary sensory neurons in porcine spinal cord[14]. Furthermore, the presence of ET-1 mRNA has also been demonstrated by *in-situ* hybridization in these neurons in humans[15]. This, together with the presence of specific binding sites for ET-1 and the pharmacological activities of exogenously applied ET-1 in the CNS[8,9,14], suggests a possible role for ET-1 as a novel neuromodulator molecule.

Multiple receptor subtypes?

The existence of three endothelin isopeptides also opens up the possibility of the existence of multiple endothelin receptor subtypes. ET-3 is in general a much weaker vasoconstrictor than ET-1 or ET-2. However, whereas ET-3 has only 25–50% the activity of ET-1 as a pressor agent *in vivo*, it produces a significantly more profound initial transient depressor response than ET-1 when injected (1 nmol kg^{-1}, i.v.) into anesthetized rats[12]. In isolated perfused mesentery, the activity of ET-3 is

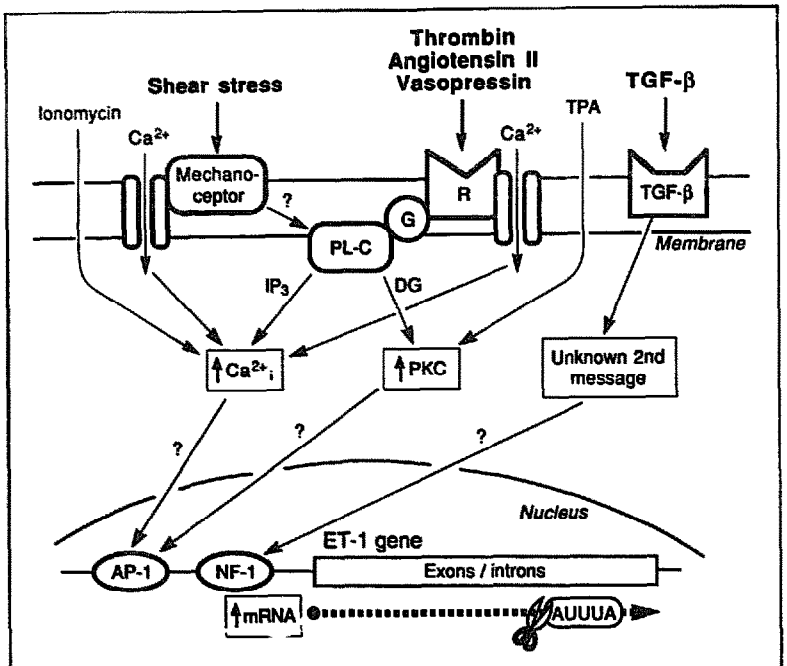


Fig. 2. Possible regulatory mechanisms of ET-1 production in endothelial cells. See text for detailed explanation. **R**, *Receptor molecules that belong to the 'rhodopsin-type' superfamily;* **G**, *G proteins;* **PL-C**, *phospholipase C;* **PKC**, *protein kinase C;* **TGF-β**, *transforming growth factor β;* **TPA**, *12-O-tetradecanoylphorbol 13-acetate;* **IP_3**, *inositol-1,4,5-trisphosphate;* **DG**, *1,2-diacylglycerol;* **AP-1** *and* **NF-1**, *transcription factors.*

about 10% that of ET-1 in constricting the unconstricted vascular bed, whereas both peptides are approximately equipotent in relaxing the preconstricted mesentery via the formation of EDRF (Ref. 16). One interpretation of these findings is that the endothelin receptors in vascular smooth muscle cells and endothelial cells are different: a receptor with a higher affinity for ET-1 may transduce the vasoconstrictor signal in smooth muscle cells, whereas a receptor with higher affinity for ET-3 may be involved in the endothelin-induced EDRF release in endothelial cells. These pharmacological findings are supported by detailed radioligand binding studies with all three ^{125}I-labeled isopeptides[17] which have shown that chick cardiac membrane contains two distinct subpopulations of endothelin binding sites: one has higher affinity for ET-1 and ET-2 than for ET-3; the other has higher affinity for ET-3 than for ET-1 or ET-2.

Structure–activity relationships

Like many other disulfide-containing bioactive peptides, destruction of the intrachain disulfides and/or the loop structures of ET-1 results in a marked decrease of its vasoconstrictor activity[18]. Reduction and alkylation of all four Cys residues reduces the molar potency of ET-1 as a constrictor of porcine coronary artery by a factor of more than 100. The outer disulfide bridge (Cys1–Cys15) appears to be more important than the inner disulfide (Cys3–Cys11); substitution of Cys1 and Cys15 with Ala residues reduces the potency of ET-1 by a factor of more than 200, whereas similar substitution of Cys3 and Cys11 results in a reduction of the potency by a factor of only about 20 (Ref. 19).

Alteration of the peptide chain length also considerably affects the vasoconstrictor potency of ET-1. The potency of ET-1 decreases by a factor of more than 1000 as a result of the removal of the C-terminal Trp21 (Ref. 18). Further deletion of the C-terminal tail to produce ET-1_{1-19} results in an additional reduction of constrictor activity by a factor of 100. The constrictor activities of the porcine 39 amino acid precursor big ET-1, and its shorter derivative big ET-1_{1-25} are also considerably less than that of mature ET-1_{1-21}; the molar potencies of porcine big ET-1_{1-39} and big ET-1_{1-25} in constricting isolated vascular strips are less than that of ET-1_{1-21} by factors of 100–140 and 50, respectively[20,21]. Moreover, α-amidation of the C terminus of ET-1_{1-21} results in a reduction of activity by a factor of 40 (Ref. 22). Thus the peptide chain of mature ET-1 (21 amino acids with a free C-terminal α-carboxyl) may be the optimum length with a maximal vasoconstrictor potency.

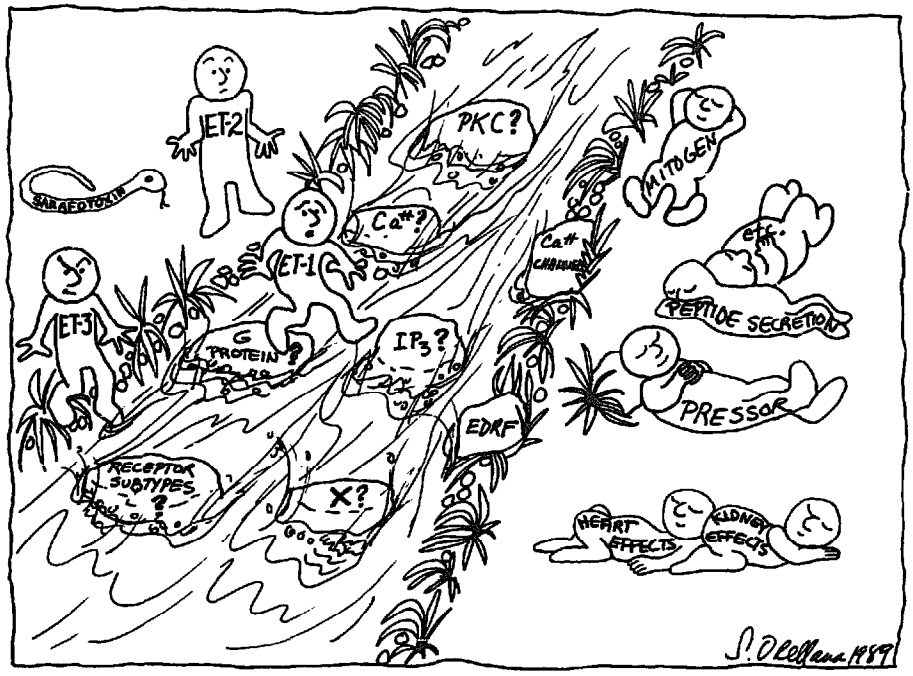
The fact that the proteolytic processing at Trp21–Val22 increases the activity of the peptide by more than 100-fold illustrates the physiological importance of the conversion of big ET-1_{1-39} into ET-1_{1-21}. The conditioned culture medium from porcine aortic endothelial cells contains both mature ET-1_{1-21} and big ET-1_{22-39} (including its truncated derivative big ET-1_{23-39}) at approximately equimolar concentrations, together with a very small amount of big ET-1_{1-39} (Ref. 23). This provides direct evidence for the hypothesis that ET-1_{1-21} is produced from big ET-1_{1-39} via an endoproteolytic processing rather than by a carboxy-exopeptidase reaction. The putative endothelin-converting enzyme involved in this endoproteolysis may provide an important target for pharmacological intervention.

Regulation of production

One frustrating feature in endothelin biology is the lack of a positive demonstration that endothelin released *in situ* mediates endothelium-dependent vasoconstriction. The ultrastructural observation that endothelial cells contain almost no dense secretory granules in which presynthesized active substances such as peptides could be stored led us to speculate that the secretion of ET-1 from endothelial cells is regulated at the level of peptide synthesis (i.e. transcription and/or translation) and not at the level of the release mechanism[2]. It may therefore be necessary to develop new methods that can detect responses with very slow time-courses (at least hours) before it will be possible to detect endothelin-mediated constrictor responses.

Nevertheless, a recent report

has described an endothelin-like vasoconstrictor peptide in effluents from perfused guinea-pig lungs[24]. The amount of this peptide in the venous effluents increases by three- to fivefold after infusion of thrombin or the Ca^{2+} ionophore calcimycin. It remains to be established, however, whether this increased secretion of endothelin contributes to the previously described constrictor response of this vascular bed to thrombin.



The induction of ET-1 mRNA and/or peptide by various chemical and mechanical stimuli has been studied mainly in cultured endothelial cells (Fig. 2). The growing list of physiological stimuli that can increase ET-1 production includes, so far, thrombin[2], transforming growth factor β (TGF-β; Ref. 25), angiotensin II (Ref. 26), [Arg]vasopressin[26] and fluid dynamical shear stress[27]. Thrombin, angiotensin II and vasopressin stimulate phospholipase C activity in endothelial cells, leading to the formation of the second messengers inositol 1,4,5-trisphosphate (which mobilizes Ca^{2+} from intracellular storage sites) and 1,2-diacylglycerol (which stimulates protein kinase C). Shear stress also increases intracellular free Ca^{2+} concentration in cultured endothelial cells by both stimulating the influx of extracellular Ca^{2+} and mobilizing intracellular Ca^{2+} (Ref. 28). Indeed, ET-1 mRNA and peptide are also induced by Ca^{2+} ionophores and phorbol esters[26,29]. These observations are consistent with the idea that the production of ET-1 in endothelial cells can be regulated by intracellular Ca^{2+} and by protein kinase C – that is, possibly by PI turnover signaling in endothelial cells.

The 5′ promoter region of human ET-1 gene contains several elements responsive to 12-*O*-tetradecanoylphorbol 13-acetate (AP-1/jun-binding elements) which are found in other genes that can be induced by phorbol esters[30]. It also contains the nuclear factor-1-binding elements[30] that have recently been recognized to be involved in gene regulation in response to TGF-β. Whether these potential regulatory DNA elements in the ET-1 gene are actually involved in the regulation of ET-1 production by agents such as those listed above will be determined by promoter mapping studies.

The level of ET-1 mRNA in endothelial cells may be controlled not only by transcriptional regulation but also by post-transcriptional regulation of mRNA degradation. Half-life studies using the transcription inhibitor actinomycin D have revealed that ET-1 mRNA is extremely labile, having an intracellular half-life of about 15 min (Ref. 29). This rapid degradation is specific to ET-1 mRNA, since β-actin mRNA has a much longer half-life (10–20 h) in the same cells. Both human and porcine ET-1 mRNAs possess several 'AUUUA' sequences in the 3′ non-translated regions. These AU motifs have been recognized to be involved in highly selective mRNA destabilization and are found in mRNAs encoding certain transiently expressed cytokines, growth factors and nuclear proto-oncogene products (usually those involved in programming of cellular growth and differentiation). It is conceivable that ET-1 may also belong to this class of signaling molecule.

Metabolism

Pharmacokinetic studies in rats show that i.v. injected ^{125}I-labeled ET-1 is rapidly eliminated from the bloodstream, with an initial half-life of less than two minutes[31,32]. This rapid decay rate is virtually unchanged even when a pressor amount (1–2 nmol kg^{-1}) of cold ET-1 is co-administered with the radiolabeled tracer. Nevertheless, the pressor response usually lasts more than 1 h. The extremely slow dissociation of ET-1 from its receptors on vascular smooth muscle cells[33] may at least partly account for the discrepancy between the time-course for elimination of the exogenously applied peptide from circulating blood and that for the pressor effect of the peptide. Within a few minutes of i.v. injection of ^{125}I-labeled ET-1, a major fraction of the radioactivity is found in the lungs, kidneys, liver and spleen[9,32]. Moreover, HPLC analysis of the injected ^{125}I-labeled ET-1 remaining in the circulating blood indicates that its chemical form is not significantly altered in the bloodstream[31,32]. ^{125}I-labeled ET-1 also appears to be very stable in heparinized whole blood *in vitro* at 37°C. Thus the rapid elimination of ET-1 from the circulation may be mainly due to the trapping of the intact peptide by the parenchymal organs. It remains to be elucidated whether a specific peptidase(s) inactivating and metab-

olizing endothelins is present in these endothelin-clearing tissues, as well as in the biological target tissues and/or cells.

Possible mechanisms of action

The putative specific receptor for ET-1 has recently been solubilized from chick cardiac membrane[34]. The ^{125}I-labeled ET-1 binding activity can be dissociated from the binding activity for the dihydropyridine-derivative [^{3}H]PN 200-110 by sucrose gradient ultracentrifugation. Further, the ^{125}I-labeled ET-1 binding protein is not immunoprecipitated with a monoclonal antibody that recognizes dihydropyridine-sensitive Ca^{2+} channel complex. Affinity cross-linking studies show that ^{125}I-labeled ET-1 binds to a protein species with an apparent M_r of about 50000 in SDS gels[34]. Taken together with the fact that ET-1 can stimulate phospholipase C via a G protein (see below), this supports the speculation that (at least one) ET-1 receptor belongs to the G protein-coupled, rhodopsin-type receptor superfamily.

ET-1 stimulates phospholipase C in many systems including cultured vascular smooth muscle cells[35] and fibroblasts[36], and isolated intact vascular smooth muscle tissues[37]. In A10 rat aortic smooth muscle cells, stimulation of phospholipase C by ET-1 is mediated by a pertussis toxin-insensitive G protein (Y. Takuwa *et al.*, unpublished). The mitogenic effect of ET-1 in quiescent Swiss 3T3 fibroblasts is abolished after the depletion of protein kinase C by a long-term pretreatment of the cells with phorbol ester, indicating that activation of protein kinase C by ET-1 via stimulation of PI turnover response may be essential for the mitogenic effect of ET-1 (Ref. 36).

Currently available data including those mentioned above demonstrate that ET-1 does not act directly on the dihydropyridine-sensitive Ca^{2+} channels as we originally hypothesized. However, although in some systems ET-1 acts almost completely independently of the voltage-dependent Ca^{2+} channel[38], in others it appears to be closely (but not directly) associated with the activation of the Ca^{2+} channels. ET-1 markedly augments dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel current in the whole-cell clamping of freshly dispersed smooth muscle cells from porcine coronary artery[39]. The ability of ET-1 applied outside the patch to increase single-channel Ca^{2+} currents in the cell-attached patch-clamp recording indicates that the peptide acts via a readily diffusible second messenger[40].

ET-1 significantly increases the contractile sensitivity of partially depolarized porcine coronary artery strips to extracellular Ca^{2+}. Constriction of the artery to ET-1 is effectively suppressed, though not abolished, by nanomolar concentrations of dihydropyridine Ca^{2+} antagonists[39], and the response to less than 3–5 nM of ET-1 absolutely requires the presence of extracellular Ca^{2+} in this artery (although higher concentrations of ET-1 can cause contractions independently of extracellular Ca^{2+}). The inhibition by ET-1 of renin secretion from juxtaglomerular cells, ET-1-induced stimulation of catecholamine release from adrenomedullary cells, and ET-1-mediated substance P release in isolated spinal cord (see Table I) have also been reported to be dependent on extracellular Ca^{2+} and sensitive to dihydropyridine Ca^{2+} antagonists. The precise mechanisms of the indirect coupling of the ET-1 receptor to the Ca^{2+} channel remain to be elucidated.

The currently available data suggest that the signal transduction at the ET-1 receptor in vascular smooth muscle cells may be at least qualitatively similar to those for other well known vasoconstrictor peptides such as angiotensin II and vasopressin. But can the extremely long-lasting and slow-onset vasoconstriction induced by endothelins be explained entirely by the existing dogma of receptor mechanisms? One attractive possibility is that, as in the case of the subtypes of catecholamine and muscarinic receptor, the signal transduction pathways differ from one receptor subtype to another.

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