Cellular signaling by peptides of the endothelin gene family

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ABSTRACT

Endothelins (ET) are a family of regulatory peptides synthesized by selected endothelial and epithelial cells that act in a paracrine fashion on nearby smooth muscle or connective tissue cells. We review the pathways of transmembrane signaling triggered by binding of endothelin peptides to receptors on the plasma membrane. Although our understanding of many components is unclear, endothelin peptides appear to evoke a phosphoinositide-linked signaling system that bears a striking resemblance to signaling pathways activated by other regulatory peptides. Expression of endothelin receptors and specific pathways stimulated by activated receptors are controlled in a cell- and tissue-specific manner, which perhaps explains the diverse biological actions of endothelin in different tissues. Complex negative feedback pathways regulate endothelin-induced signaling at the receptor and second messenger levels. Moreover, by regulating the activity of sequence-specific DNA binding proteins, short-term signals by ET can be extended to long-term effects involving gene expression. Regulation of gene expression by ET could account for complex events such as mitogenesis and vascular and tissue remodeling in disease. — SIMONSON, M. S.; DUNN, M. J. Cellular signaling by peptides of the endothelin gene family. *FASEB J.* 4: 2989–3000; 1990.

Key Words: endothelium-derived mediators • regulatory peptides • phosphoinositide cascade • cytosolic free [Ca^{2+}] • protein kinase C

OVERVIEW

The recent discovery of ET[2] by Yanagisawa et al. (1) proved that the endothelium elaborates a potent vasoconstrictor peptide (2–4). During the past 2 years many laboratories have analyzed the structure, expression, and function of ET. These studies revealed that ETs are a family of homologous peptides with varied biological actions, particularly those involved with vasoconstriction/smooth muscle contraction; the physiology and pathophysiology of cardiac, pulmonary, and renal function; and mitogenesis and tissue remodeling. In this review we analyze the signaling pathways by which ET mediates such diverse biological actions. Limitations of space preclude a full treatment of ET-induced biological events, which have recently been reviewed (5–8). Instead we shall focus our discussion primarily on short-term transmembrane signaling. We shall also suggest how the molecular mechanisms for short-term actions might be expanded to explain long-term ET effects.

ENDOTHELIN: A PRIMER

Structure of ET isopeptides

ETs are a family of acidic, 21-amino-acid peptides (≈2.5 kDa) found in at least four distinct isoforms: ET-1, ET-2, ET-3, and VIC (vasoactive intestinal contractor or endothelin β, mouse) (1, 9–12). The discovery of VIC (12), which is expressed predominately in the intestine, raises the possibility of some organs expressing tissue-specific ET variants. As illustrated in **Fig. 1,** ET isopeptides are highly homologous and share a common design including *1*) two disulfide bonds (Cys^1-Cys^{15} and Cys^3-Cys^{11}), *2*) a cluster of polar, charged side chains residing within a hairpin loop (residues 6–10), and *3*) a hydrophobic C terminus (residues 16-21) containing the aromatic indole side chain at Trp^{21} (1, 9, 11).[3] Total synthesis of ET was

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[2]Abbreviations: ET, endothelin; VIC, vasoactive intestinal contractor; PtdIns, phosphatidylinositol; DHP, dihydropyridine; PLA_2, phospholipase A_2; PG, prostaglandin; VOC, voltage-operated channel; ROC, receptor-operated channel; SRE, serum response element; CRE, cAMP response element; FBS, fetal bovine serum; APR, acute phase reactant; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, intracellular free $[Ca^{2+}]$; pH_i, intracellular pH.

[3]Nomenclature: For naming ET isopeptides, we use the convention suggested by Masaki and Yanagisawa (see ref 5) where ET-1 refers to the sequence originally described as human or porcine, and ET-3 refers to the peptide originally described as rat ET. All mammalian genomes examined to date carry genes encoding ET-1, -2, -3, and VIC.

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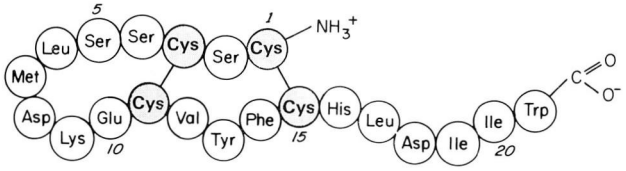
achieved by mixed solid-phase and solution chemistry with selective cross-linking of the four Cys residues (13). Bioassay measurements with synthetic ET-1 variants reveal the following structure-activity relationships (14). Reduction and alkylation of disulfide bonds displace the ED_{50} for smooth muscle contraction 3 log units to the right. Deletion of Trp^{21} displaces the ED_{50} by 3–4 log units, whereas deletion of residues 17-21 causes a complete loss of activity. Last, cleavage of Lys^{9} by lysl-endopeptidase causes a three order of magnitude loss of contractile activity. These experiments demonstrate that the hydrophobic C terminus is required for bioactivity and that the loop configuration is also important.

ET peptides share sequence homology (≈67%, Fig. 1) and bioactivity with sarafotoxins, peptide toxins isolated from venom of *Atractaspis engaddensis* (15), and it seems likely the two peptide families share a common evolutionary origin (16). The high degree of sequence homology between ET and sarafotoxins, and between ET isopeptides in mammals, suggests that ET genes have evolved under strong pressure to conserve the structure and function of mature ET peptides. Thus genes encoding a snake venom toxin appear to have evolved into genes encoding an important mammalian regulatory peptide.

Biosynthesis of ET

In a manner analogous to other hormones and neurotransmitters, ET isopeptides arise via proteolytic processing of isopeptide-specific prohormones (**Fig. 2**).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
ET-1	C	S	C	S	S	L	M	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
ET-2	C	S	C	S	S	W	L	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
ET-3	C	T	C	F	T	Y	K	D	K	E	C	V	Y	Y	C	H	L	D	I	I	W
VIC	C	S	C	N	D	W	L	D	K	E	C	V	Y	F	C	H	L	D	I	I	W
S6b	C	S	C	K	D	M	T	D	K	E	C	L	Y	F	C	H	Q	D	V	I	W

Figure 1. Structure and sequence homology of ET isopeptides. Top) Proposed conformation of mature, 21-amino-acid ET peptides with hairpin loop and extended C terminus. Bottom) Sequence homology of the known ET isopeptides. For comparison, the sequence of a single sarafotoxin peptide (S6b) is given. Sarafotoxins are most likely the evolutionary precursors of ET. VIC, vasoactive intestinal contractor.

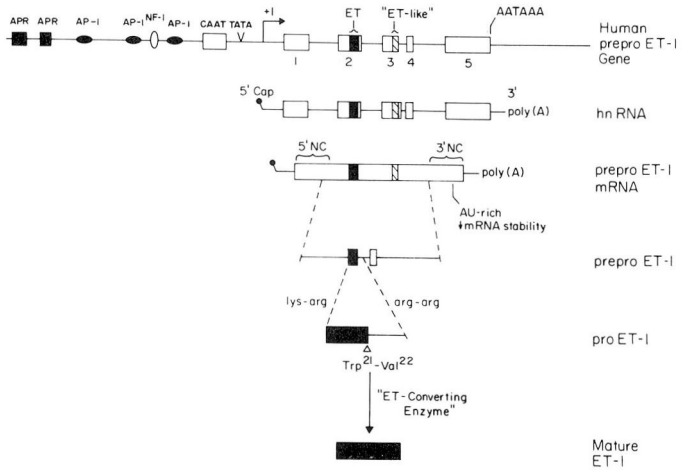


Figure 2. Schematic representation of the structural organization of the human preproET-1 gene and its expression products. The preproET-1 gene is not drawn to scale and the RNA transcripts have been enlarged for the purpose of illustration.

The amino acid sequences of preproETs have been predicted by sequencing cDNA clones isolated from porcine aortic endothelial cell and human placenta cDNA libraries (1, 9, 11, 12), and these sequences are concordant with partial amino acid sequences derived from proteolytic fragments. PreproETs are large polypeptides (≈200 amino acids) that demonstrate species- and isopeptide-specific differences in amino acid sequence. In addition to containing the mature ET peptide, the prepro-precursors contain a cysteine-rich, ET-like region (15 residues) that is highly conserved (1, 9) (Fig. 2). The biological significance of differences in amino acid sequence between the prepro species, and of the presence of an ET-like peptide within preproET, are unclear. Co- and posttranslational modification of the preproET species are also unknown.

In cultured endothelial cells, preproET-1 is proteolytically cleaved to form a 38 (human)- or 39 (porcine)-amino-acid proET-1 (1, 6, 9) (Fig. 2). A novel protease, putatively an endothelin converting enzyme, then cleaves Trp^{21}-Val^{22} of proET-1 to form the mature ET peptide. Conversion of proET-1 to ET-1 is essential for bioactivity and presumably involves an endopeptidase with chymotrypsin-like activity. Characterization and compartmentalization of this putative ET-converting enzyme will have important implications for regulation of ET biosynthesis and for possible pharmacological blockade.

Molecular biology of the ET gene family

Evidence suggests that different ET isopeptides are encoded by distinct genes. Southern blotting (under reduced stringency) or human, rat, and porcine genomic DNA reveals separate genes encoding each isopeptide-specific preproET (11, 12). Restriction mapping suggests separate chromosomal loci for each gene (11); genetic mapping using human-mouse somatic hybrid

cell lines links the genes for preproET-1 and ET-3 to human chromosomes 3 and 20, respectively (17, 18).

Structural organization of ET genes

To understand the mechanisms and regulation of ET gene expression, genomic clones have been isolated and sequenced for the human preproET-1 and ET-3 genes (Fig. 2). The human preproET-1 gene (6.8 kb) contains five exons and four introns (17, 19). Nucleotide sequences encoding mature ET-1 are located in the second exon, and the 15-amino-acid ET-like peptide is encoded in the third exon. It seems likely that the second and third exons may derive from a common progenitor exon (17). A similar exon arrangement exists in the preproET-3 gene, leading Bloch and co-workers (18) to hypothesize that the ET-1, ET-2, and ET-3 genes evolved by gene duplication events. A search of the EMBL Data Base failed to find significantly similar sequences in other genes (except for an Alu repetitive element), implying that the preproET-1 gene is unrelated to other gene families (19). The promotor region of the preproET-1 gene contains a typical eucaryotic TATA box and CAAT sequence. In addition, 5' upstream putative *cis*-acting sequences exist for the AP-1/*jun,* NF-1, and APR *trans*-acting factors, and intragenic sequences are present for NF-1 and APR (19). To our knowledge, gene transfer studies have not been conducted to confirm gene regulation via these elements, but there is increased ET-1 gene expression after treatment with phorbol esters and TGF-β (19), which are known to activate gene expression via AP-1 and NF-1 DNA-binding transcription factors. APR sequences may mediate the induction of mRNA under physical stress in vivo, and could account for the increased expression of ET-1 observed in pathophysiological settings (see ref 6). Analysis of the 3' noncoding region of preproET-1 mRNA reveals AU-rich sequences thought to decrease mRNA stability in the cytoplasm. Consistent with this idea, the $t_{1/2}$ of preproET-1 mRNA is short ($\approx$15 min) and the mRNA transcripts are superinduced by cycloheximide (19). These findings suggest that expression of the preproET-1 gene is regulated at several levels, including transcriptional control and mRNA stability.

Tissue-specific ET gene expression

ET isopeptides are differentially expressed only in specific tissues, which suggests that tissue-specific factors control the rate of ET gene expression. There are two approaches to investigating tissue-specific ET gene expression: *1*) Northern analysis of preproET mRNA transcripts in RNA isolated from different tissues; and *2*) in situ hybridization. Northern blotting reveals that preproET-1, but not preproET-3, is expressed by cultured endothelial cells from large vessels (1, 12, 18, 19) and microvessels (20). Transcripts for preproET-1 have also been identified in RNA isolated from porcine aortic intima in vivo (1). mRNA for preproET-1 and ET-3 is abundant in fetal lung, spleen, pancreas, and to a lesser extent in fetal kidney, atrium, and ventricle (12, 18). By contrast, preproET transcripts have been more difficult to demonstrate in adult porcine and murine tissues (1, 12), but several investigators (20, 20a, 20b) have demonstrated transcripts for both preproET-1 (2.5 kb) and preproET-3 (3.7 kb) in adult rat lung, kidney, eye, and brain. ET-3 was predominately expressed in kidney, eye, and brain, whereas both ET-3 and ET-1 were abundantly expressed in the lung. In addition, mRNA transcripts (1.4 kb) for VIC have been found in the murine intestinal tract (12). By in situ hybridization with oligonucleotide probes, MacCumber et al. (20) localized ET gene expression in a variety of fetal and adult rat tissues. ET expression was highest in the lung, and at high-resolution ET mRNA was associated with small bronchioles, especially over epithelial cells. In the rat kidney, ET was expressed predominately in the medullary vasa recta (20).

Further work is necessary to identify sites of ET gene expression in vivo. The inability to demonstrate ET gene expression in some adult tissues could reflect the relative insensitivity of Northern analysis to detect rare transcripts or poor hybridization of the ET-1 and ET-3 probes with noncomplementary tissue-specific ET isoforms. It seems likely, however, that ET isopeptide genes are differentially expressed in the epithelium and endothelium of a variety of fetal and adult tissues. In addition, ET gene expression is regulated at both tissue-specific and developmental levels.

Biological actions of ET

The widespread but tightly regulated expression of ET genes in vivo suggests diverse biological actions for ET peptides. As shown in **Table 1,** ET has numerous biological actions in vitro and in vivo (see refs 5–8, 21 for review). ET is one of the most potent contractile agents yet identified, and this accounts for a number of the hemodynamic, cardiac, pulmonary, and renal effects. ET contracts both vascular and nonvascular smooth muscle and has strong inotropic and chronotropic effects on the myocardium. ET increases plasma levels of a variety of vasoactive hormones (renin, aldosterone, etc.; see Table 1) and may modulate synaptic transmission (20a, 20b). Experiments in vitro have shown that ET stimulates mitogenesis in smooth muscle and glomerular mesangial cells, and in 3T3 fibroblasts. Coupled with the fact that ET regulates expression of certain genes, the promitogenic potential of ET raises the possibility that ET contributes to vascular or glomerular remodeling in disease. There is also considerable interest in the role of ET in the pathogenesis of cardiovascular and renal disease (5, 6, 8, 21).

ET therefore appears to be involved in a large array of cellular functions. How can ET peptides perform so many functions? The following sections review our current understanding of signal transduction by ET.

TABLE 1. *Biological actions of endothelin peptides*[a]

Hemodynamic effects
Causes initial depressor response followed by sustained pressor effect (regional differences in vasoconstriction exist)
Cardiac effects
Evokes positive inotropic and chronotropic effects on myocardium
Stimulates intense vasoconstriction of coronary arteries
Neuroendocrine effects
Increases plasma levels of ANF, renin, aldosterone, and catecholamines
Modulates synaptic transmission
Renal effects
Increases renal vascular resistance
Decreases glomerular filtration rate, renal blood flow, the glomerular ultrafiltration coefficient
Increases Na^+ reabsorption through hemodynamic actions
Decreases Na^+ reabsorption through inhibition of Na^+-K^+-ATPase
Smooth muscle effects
Contracts vascular smooth muscle; veins possibly more sensitive than arteries
Contracts numerous nonvascular smooth muscles
Promitogenic effects
Stimulates mitogenesis in vascular smooth muscle cells, 3T3 fibroblasts, and glomerular mesangial cells
Gene expression
Up-regulates mRNA expression of VL30 gene and the *c-fos* and *c-myc* protoncogenes

[a] See text and references (5–8, 21) for details.

TRANSMEMBRANE SIGNALING BY ET

ET receptors

Receptor mapping in vivo: sites of action

The distribution of ET receptors in vivo has been mapped using radiolabeled ET and autoradiography (**Table 2**). Saturable, specific binding sites for [^{125}I]ET ($K_d \approx 0.5$ nM) have been identified in numerous fetal and adult organs including lung, kidney, heart, intestine, adrenal gland, eye, and brain (20, 20a, 22–24). The density of binding sites is especially high in the lung and heart (20, 23, 24). Although the overall density in the kidney is somewhat less, a high density of ET binding sites was localized in the cortex to glomeruli and to a lesser extent proximal tubules, and in the medulla to vasa recta bundles and papilla (20, 22). [^{125}I]ET binding was not displaced by Ca^{2+} channel blockers (i.e., nifedipine, etc.), peptide neurotoxins (i.e., apamin, ω-conotoxin), adrenergic agonists, or vasoconstrictors (i.e., histamine, angiotensin II, arginine vasopressin), which suggests that ET binds to a specific cognate receptor and not directly to an ion channel or other nonspecific receptor. From analysis of the radioligand binding data and the in situ hybridization experiments discussed above, we derive several conclusions regarding the cellular signaling of ET.

1) ET probably acts in diverse cells and tissues, and a neural localization of ET binding site indicates that ET might act as a neurotransmitter or neuromodulator.

2) In many tissues the proximal location of cells with ET binding sites and those expressing ET mRNA transcripts suggests that the peptide probably acts as a local hormone synthesized by endothelial or epithelial cells and communicates in a paracrine fashion with nearby smooth muscle cells, fibroblasts, or pericytes.

3) There are also regions expressing abundant binding sites but little or no mRNA (i.e., cardiac ventricular muscle or kidney glomerulus). At these sites ET might come from the bloodstream or from autonomic innervation. Alternatively, these tissues might synthesize ET isopeptides (i.e., ET-2 or VIC) whose mRNA would not cross-hybridize with the oligonucleotide probes used (20).

Further experiments with isopeptide-specific oligonucleotide probes and radiolabeled peptides should help establish whether ET acts mostly as a paracrine hormone or whether circulating levels of ET have biological significance. It is unclear whether disease alters the expression of ET receptors in vivo.

Biochemical characterization of ET binding sites in vitro

High-affinity, saturable binding sites for [^{125}I]ET isopeptides and [^{125}I]sarafotoxin have been characterized in intact cells and in membrane preparations from vascular and nonvascular smooth muscle, 3T3 fibroblasts, glomerular mesangial cells, and neurons (i.e., refs 5, 25–30). Saturation analysis demonstrates a range of apparent dissociation constants (K_d) of ≈0.1–10 nM and a high density of binding sites, especially on vascular smooth muscle. At 37°C steady states are observed within 10–20 min, whereas at lower temperatures steady states are not obtained for hours. Although there are exceptions, most K_d values are comparable to the EC_{50} values for ET-induced biological events, and the relative potency of ET isopeptides and analogs competing for binding sites is in direct proportion with their

TABLE 2. *ET receptor mapping in vivo*[a]

Organ	Comments
Lung	High density and wide distribution; especially prominent in bronchus
Kidney	In cortex, glomeruli > proximal tubules; in medulla, high density in vasa recta
Heart	High density in nerves, atria, ventricles, and smooth muscles of coronary arteries
Intestine	Localized mostly to mucosal layer
Adrenal gland	High density in zona glomerulosa; moderate density in adrenal medulla
Eye	High density in iris, localized to stroma; also high density in choroid, retina, and endothelial layer of cornea
Brain/nerves	Widespread but distinct localizations; prominent in cerebellum and grey matter of spinal cord

[a] See text and refs 5, 20, 22–24 for details.

contractile activity. Both saturation and competitive displacement binding studies suggest that multiple classes of receptors exist with different affinities for ET isopeptides and that the distribution of the receptor subtypes is tissue-specific (5, 6, 27–29). The physiological significance of ET receptor subtypes is unclear, and it remains to be determined whether these putative receptors identified by radioligand binding studies represent functional receptors. Several laboratories have reported that dissociation of [^{125}I]ET from its receptor is extremely slow, and that binding of ET to its receptor might not be entirely reversible (5, 25, 26, 30); this irreversibility complicates analysis of equilibrium and kinetic binding data and suggests a complex interaction between ET and its receptor. Slow dissociation of ET from its receptor has been speculated to account for the characteristically long-lasting vasoconstrictor activity of ET (6). The regulation of ET receptor number, activity, and turnover (by phosphorylation, internalization, etc.) is unknown; however, pretreatment of smooth muscle and mesangial cells with ET for 24 h markedly reduces ET receptor number without changing K_d (25, 30).

Purification, cloning, and sequencing of ET receptors will be critical for understanding the signal transduction of ET peptides. To this end, two laboratories have reported detergent solubilization (CHAPS) of ET/sarafotoxin receptors in an active binding conformation from human placenta and rat cerebellum (31, 32). Cross-linking of the placental receptor with [^{125}I]ET-1 revealed a specifically labeled band of ≈32 kDa (31), although other cross-linking studies of membrane preparations suggest the possibility of multiple classes of ET receptors (i.e., kDa = 34, 45 or 45, and 53) (27, 28, 32, 33).

ET evokes the phosphoinositide cascade

Stimulation of phospholipase C

The activity of phospholipase C increases rapidly after activation of ET receptors. ET stimulates a dose-dependent increase of PtdIns turnover in vascular smooth muscle cells (34–38), fibroblasts (38, 39), atrial cells (40), and glomerular mesangial cells (41, 42). Experiments demonstrating ET-activated PtdIns turnover in cultured mesangial cells are presented in **Fig. 3*A***. ET also elevates phospholipase C activity when applied to intact preparations of coronary artery strips (43) and aorta (44). Sarafotoxin stimulates PtdIns turnover in rat atrial and cerebellar slices (15), suggesting that ET and sarafotoxins share similar pathways of signal transduction in mammals (16). As expected, in target cells phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to form the water-soluble (Ins(1,4,5)P_3) and the neutral diacylglycerol, two second messengers of the phosphoinositide cascade, which in turn elevate $[Ca^{2+}]_i$ and activate protein kinase C (see below). Depending on the phosphatase/kinase activities of the target cells, a variety of inositol phosphates is formed. In mesangial cells, for example, abundant levels of Ins(1,3,4,5)P_4 are formed from Ins(1,4,5)P_4, presumably via an Ins(1,4,5)$P_3$3-kinase activity (Fig. 3*B*) (41). In many cells Ins(1,4,5)$P_3$3-kinase is a Ca^{2+}-sensitive enzyme (45), and it is likely that the initial increase in $[Ca^{2+}]_i$ triggered by ET determines the pathway by which Ins(1,4,5)P_3 is ultimately metabolized. Ins(1,3,4,5)P_4 might play a synergistic role with Ins(1,4,5)P_3 to elevate $[Ca^{2+}]_i$ in these cells (7, 45). Ins(1,4)P_2 and Ins(1,3,4)P_3 result from 5-phosphomonoesterase-catalyzed dephosphorylation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, respectively (Fig. 3), but these products are probably inactive and are ultimately metabolized to free inositol. Similar patterns and kinetics of inositol phosphate formation have been demonstrated in other cell types after agonist stimulation of phospholipase C (45). Preliminary experiments suggest that a pertussis toxin-sensitive G protein couples ET receptors to phospholipase C. In vascular smooth muscle cells, pretreatment with pertussis toxin attenuates ET-stimulated InsPtd turnover (46), and in rat mesangial cells not only did pertussis toxin inhibit ET-induced InsPtd turnover but GTPγS had a potentiating effect (C. Thomas and M. J. Dunn, unpublished results). By contrast, pertussis toxin fails to inhibit InsPtd turnover in rat-1 fibroblasts and A-10 smooth muscle cells stimulated with ET (38), implying that multiple G proteins couple ET receptors to phospholipase C.

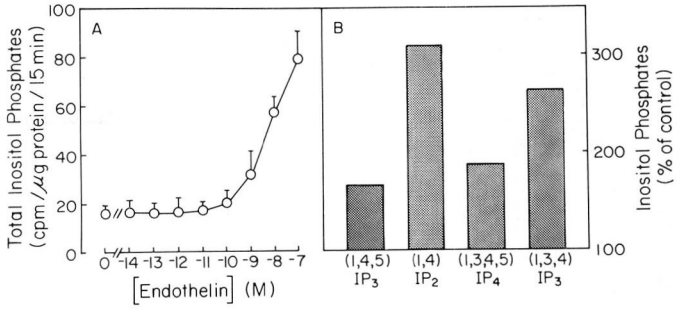


Figure 3. ET-1 activates phospholipase C in cultured rat mesangial cells. *A*) ET-1 stimulates a dose-dependent increase in turnover of total InsPtd in the presence of lithium. *B*) HPLC analysis of water-soluble InsPtd isomers produced 30 s after addition of 0.1 μM ET-1. From ref 7 by permission.

Although it is too early to conclude whether the ET receptor is channel-linked, catalytic, or G protein-linked, the evidence accumulated suggests that ET initially activates a membrane transduction process comprising a cell-surface receptor, a coupling G protein and phospholipase C. The consequences of activating this ubiquitous signal transduction pathway are discussed below.

ET stimulates protein kinase C activity

In both smooth muscle cells (38, 47, 48) and fibroblasts (38), ET rapidly stimulates a dose-dependent, biphasic increase in diacylglycerol that is sustained for 20 min or

longer. ET stimulates phosphorylation of an acidic, 76-kDa protein (a protein kinase C substrate), and 24 h pretreatment with phorbol esters, which down-regulates protein kinase C activity, inhibits phosphorylation of this protein (47). Translocation of cytosolic protein kinase C activity to the plasma membrane is also consistent with activation of protein kinase C by ET (48).

ET-induced Ca^{2+} signaling

ET-1 evokes multiple pathways to produce a robust and sustained increase in $[Ca^{2+}]_i$ in target cells (i.e., refs 7, 36, 37, 41, 42, 49, 50). As illustrated in **Fig. 4** for ET-2, ET peptides usually cause a biphasic increase in $[Ca^{2+}]_i$ consisting of a rapid (2–5 s), transient increase followed by a lesser but sustained increment. The sustained phase of $[Ca^{2+}]_i$ is especially pronounced, lasting up to 20 min in some experiments (7, 41). In glomerular mesangial cells from humans and rats, Ca^{2+} signaling appears to be a universal response to all ET isopeptides and sarafotoxin, except perhaps ET-3 (49, 50); ET-1, ET-2, and S6b stimulate similar biphasic $[Ca^{2+}]_i$ waveforms with ET-1 ≈ ET-2 > S6b > > ET-3. ProET-1 fails to elevate $[Ca^{2+}]_i$, which suggests that the conformation of the mature ET peptide (21 residues) is constrained in an inactive configuration within the precursor peptide and that proteolytic processing of proET-1 is essential to activate Ca^{2+} signaling (49). We have also observed that in human mesangial cells ET-1 and ET-2 cause oscillations of $[Ca^{2+}]_i$ that had not been previously observed in these cells (50). The most likely explanation for ET-induced $[Ca^{2+}]_i$ oscillations is that the $Ins(1,4,5)P_3$-insensitive Ca^{2+} stores trigger a wave of Ca^{2+}, which then propagates by a process of Ca^{2+}-mediated Ca^{2+} diffusion. In some cells sufficient Ca^{2+} flows through gap junctions to trigger a Ca^{2+} wave in neighboring cells, as has previously been observed in cardiac trabeculae and ciliated tracheal epithelium (see ref 45). In this way the binding of ET to mesangial cells might recruit a local population of mesangial cells to function in unison.

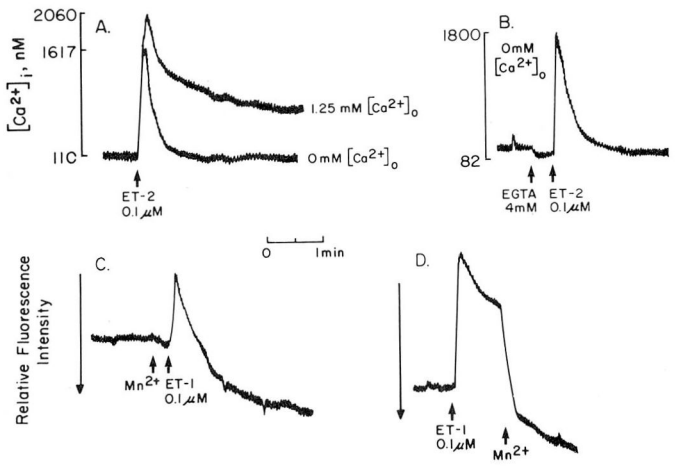


Figure 4. Ca^{2+} signaling evoked by ET-2 in cultured mesangial cells loaded with the Ca^{2+} sensitive probe, fura-2. *A*) ET-2 was added to mesangial monolayers in the presence and absence of extracellular Ca^{2+}. $[Ca^{2+}]_i$ waveforms from consecutive coverslips were superimposed to facilitate comparison. *B*) Mesangial cells incubated in Ca^{2+}-free medium were treated for 30 s with EGTA before adding ET-2. The resultant waveform demonstrates that the spike increase in $[Ca^{2+}]_i$ results primarily from release of intracellular stores. *C, D*) Experiments using Mn^{2+} to assess ET-mediated $Mn^{2+}/(Ca^{2+})$ entry. Mn^{2+} was added either before (*C*) or after (*D*) ET-1 was added; the rate of fura-2 quenching reflected Mn^{2+} entry.

Ligand receptor-activated increases in $[Ca^{2+}]_i$ result from the release of Ca^{2+} from intracellular stores, increased net influx of Ca^{2+} across the plasma membrane, or a combination of both mechanisms (45). Three lines of evidence suggest that both mechanisms contribute to ET Ca^{2+} signaling (Fig. 4). First, ET-induced $[Ca^{2+}]_i$ transients are attenuated and the sustained increase is abolished in Ca^{2+}-free medium (41). Second, pretreatment with Ca^{2+} chelators such as EGTA to chelate $Ca^{2+}{}_o$ before addition of the ET agonist similarly inhibits the spike increase and blocks the sustained phase. Last, when mesangial cells are loaded with the intracellular Ca^{2+} chelator, BAPTA, and incubated in Ca^{2+}-free medium, both ET-1 and ET-2 fail to increase $[Ca^{2+}]_i$ (41, 49, 50). Thus after binding of ET, both intracellular release and extracellular influx of Ca^{2+} contribute to the transient phase, whereas extracellular influx is primarily responsible for the sustained phase. $Ins(1,4,5)P_3$ probably mediates the release of Ca^{2+} from the intracellular stores, the endoplasmic reticulum, or calciosome (7, 45).

In phosphoinositide-linked Ca^{2+} signaling systems, protein kinase C can either inhibit or enhance the Ca^{2+} signal, depending on the net expression of feedback mechanisms activated by specific agonists. It appears that in mesangial cells inhibition of ET-stimulated Ca^{2+} signaling is an important consequence of protein kinase C activation (49–51). Pretreatment with phorbol esters or diacylglycerol analogs reduces the increase in $[Ca^{2+}]_i$ by ET. Down-regulation of protein kinase C by pretreatment with phorbol esters amplifies ET-induced $[Ca^{2+}]_i$ waveforms in both human (50) and rat (49) mesangial cells. Three major mechanisms are thought to account for the inhibitory effect of protein kinase C on Ca^{2+} signaling: *1*) inhibition of $PtdIns(4,5)P_2$ hydrolysis, thus reducing the production of $Ins(1,4,5)P_3$; *2*) activation of Ca^{2+} pumps to remove Ca^{2+} from the cytosol; or *3*) stimulation of $Ins(1,4,5)P_3$-5-phosphatase. In vascular smooth muscle cells, pretreatment with phorbol ester inhibits ET-stimulated $[^3H]InsPtd$ turnover (46), but to our knowledge experiments have not been conducted to test the contribution of other mechanisms to the down-regulation of ET Ca^{2+} signaling by protein kinase C. Given the robust increases in $[Ca^{2+}]_i$ caused by ET, this will be an important area for future investigation.

ET-induced Ca^{2+} entry: are two Ca^{2+} channels involved?

Two main types of Ca^{2+} channels regulate Ca^{2+} influx, VOC and ROC, which are opened either directly through a receptor or indirectly through some internal diffusible second messenger. Experiments designed to

elucidate the mechanism of ET-stimulated Ca^{2+} influx have produced divergent results. It was initially reported that ET promotes Ca^{2+} influx via a DHP-sensitive Ca^{2+} channel (1). Evidence that ET activates VOC comes from studies showing that DHP channel blockers inhibit ET-induced contraction (5, 21, 34, 36, 52) as well as increments in $[Ca^{2+}]_i$ (52) and $^{45}Ca^{2+}$ uptake (36) in vascular smooth muscle cells. Direct measurement of Ca^{2+} conductance by patch-clamp techniques is also consistent with activation of VOC by ET (34, 52, 53, 53a). As shown in **Fig. 5,** ET increases the probability of channel opening with a characteristically slow augmentation of channel activity; the amplitude distribution and opening time are not significantly altered (53). Using the whole cell-attached configuration, Goto et al. (52) were unable to conclude whether ET directly or indirectly activates the channel. However, the observation by Silberberg et al. (53) and Inoue et al. (53a) that bath-applied ET increases the activity of an L-type Ca^{2+} channel in the cell-attached mode strongly argues that ET acts via a diffusible, second messenger. Further evidence for indirect action comes from studies in which ET failed to displace binding of four chemically distinct L-type channel ligands (22–29, 34). In vascular smooth muscle ET depolarizes membrane potential by opening a nonselective cation channel, which in turn gates a VOC Ca^{2+} channel (34). These data are most consistent with an indirect, modulatory action of ET on L-type VOC Ca^{2+} channels, which as shown in Fig. 5 dramatically increases Ca^{2+} conductance.

In contrast, several lines of evidence suggest that ET also gates an ROC. First, in some systems contraction in response to ET is unaffected or minimally inhibited by DHP and phenylalkylamine channel blockers (21, 44, 54, 62). Second, ET-induced increments in $[Ca^{2+}]_i$ are insensitive to blockade by DHP channel blockers (41, 42, 55), even when the cells express readily activated VOC (55). Using mesangial cells loaded with the Ca^{2+}-sensitive dye, fura-2, we have used fluorescence techniques to demonstrate that ET rapidly gates a Ca^{2+} channel distinct from DHP-sensitive VOC Ca^{2+} channels (49, 50). By measuring the rate of fura-2 quenching by exogenously applied Mn^{2+}, a probe for Ca^{2+}-permeable ROC, we found that ET isopeptides rapidly increased transmembrane divalent cation permeability under conditions where these cells express minimal or undetectable VOC Ca^{2+} channel activity. ET-1 rapidly ($\leq$5 s) stimulated Mn^{2+} entry (Fig. 4*C*), and addition of Mn^{2+} after ET-1 demonstrated enhanced divalent cation uptake (Fig. 4*D*). ET-1-activated Ca^{2+} entry occurred in mesangial cells that were BAPTA-clamped to block the increase of $[Ca^{2+}]_i$ due to release from intracellular stores (49). Moreover, pretreatment with phorbol esters and diacylglycerol analogs failed to elevate $[Ca^{2+}]_i$, and in mesangial cells depleted of protein kinase C by prolonged phorbol ester pretreatment, $[Ca^{2+}]_i$ waveforms in response to ET-1 appeared to be amplified (49–51). These experiments suggest that release of Ca^{2+} from intracellular stores and activation of protein kinase C fail to mediate Ca^{2+} channel activation by ET in mesangial cells. In addition, direct measurements of Ca^{2+} conductance by patch-clamping reveal that ET increases nifedipine-insensitive Ca^{2+} channel activity, confirming that ET promotes multiple pathways of Ca^{2+} entry in a single cell (53a). Thus in addition to modifying voltage-sensitive Ca^{2+} influx, ET can also activate voltage-insensitive Ca^{2+} influx pathways, but because at present so little is known about the specific properties of ROC Ca^{2+} channels, the action of ET remains difficult to characterize.

Further work is needed to understand the pathways of Ca^{2+} influx activated by ET. Patch-clamp studies to elucidate the mechanisms by which ET modulates L-type Ca^{2+} channel activity should be especially useful in this regard. It appears, however, that ET does not directly gate VOC Ca^{2+} channels and that multiple Ca^{2+} channels are involved in cellular signaling by ET peptides. The coexistence or tissue-specific expression of DHP-sensitive and DHP-insensitive pathways of Ca^{2+} influx could explain the divergent effects of DHP Ca^{2+} channel antagonists on ET-induced contraction.

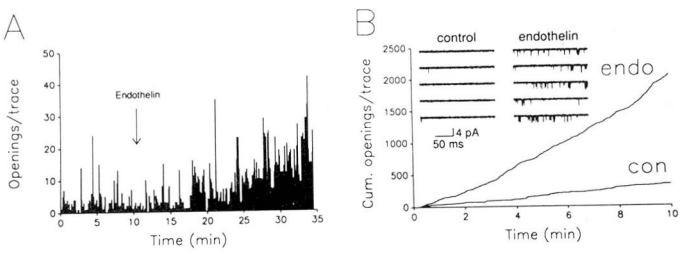


Figure 5. ET-1 increases Ca^{2+} channel activity when applied to arterial smooth muscle cells. Ca^{2+} conductance was monitored by patch-clamp techniques in the cell-attached mode. *A*) Number of channel openings per 200 ms of each depolarization. *B*) Ability of ET to increase cumulative channel openings as a function of time. From ref 53 with permission.

Biological significance of ET and the phosphoinositide cascade

Ca^{2+} is a universal second messenger involved in diverse cellular processes including motility, secretion, and ion transport. Whether initiated by pharmacomechanical or excitation-contraction coupling, most contractile processes require an increase in $[Ca^{2+}]_i$ to regulate actin-myosin interactions and generate tension. An increase in $[Ca^{2+}]_i$ is almost certainly one, but perhaps not the only signal mediating the contractile action of ET on vascular and nonvascular smooth muscle (34, 36, 43, 44) and the inotropic action on cardiac atria (40). Ca^{2+} also appears to mediate the motile effect of ET on other contractile cells such as glomerular mesangial cells (58). An increase in $[Ca^{2+}]_i$ is a good candidate to mediate the secretory effects of ET (see Table 1), particularly the increase in circulating hormones and modulation of neurotransmitter function (e.g., see refs 5, 6, 21). Ca^{2+} has a prominent gating effect on numerous ion channels including K^+, Ca^{2+}, and nonspecific ion channels, and it will be interesting to determine whether ET alters transmembrane ion permeability through these mechanisms. Van Renterghem et al. (34) have already reported that the increase in

$[Ca^{2+}]_i$ by ET gates a Ca^{2+}-sensitive K^+ channel in vascular smooth muscle.

The role of protein kinase C in ET-induced transmembrane signaling is uncertain. As discussed above, protein kinase C appears to inhibit ET-induced Ca^{2+} signaling, thereby serving as a negative feedback signal. Protein kinase C has also been implicated in the promitogenic actions of ET (6, 7, 21, 41, 42) and in the ability of ET to activate members of the immediate-early response genes (see below). Linking pathways of the phosphoinositide cascade to ET-induced biological actions will be an active area of future research.

ET stimulates the arachidonic acid cascade

Stimulation of phospholipase A_2

The first evidence that ET activates PLA_2 came from experiments by De Nucci and co-workers (56) who showed that ET-1 caused the sustained release of PGI_2 and TxA_2 from isolated preparations of guinea pig and rat lung. Activation of PLA_2 in cultured smooth muscle (46, 57) and mesangial cells (58) was subsequently demonstrated by adding ET-1 to cells that were equilibrium labeled with [^{3}H]arachidonic acid; ET rapidly stimulates sustained release of [^{3}H]arachidonate into the medium, and in vascular smooth muscle cells the arachidonic acid was derived from both phosphatidylcholine and phosphatidylinositol (57). ET-releasable arachidonic acid is then converted into prostaglandins or thromboxane depending on the enzymatic capabilities of target cells. In mesangial cells, for example, ET increases accumulation of both the vasodilatory metabolite PGE_2, and lesser amounts of the vasoconstrictor metabolites $PGF_{2\alpha}$ and TxA_2 (58). It is not known whether ET activates PLA_2 directly via a G protein or indirectly by increasing $[Ca^{2+}]_i$ (59). Experiments in vascular smooth muscle cells indicate that ET might activate phospholipase C and A_2 by parallel but independent mechanisms (46).

Biological significance of ET and the arachidonic acid cascade

Prostaglandins likely act as negative feedback signals to attenuate vasoconstriction by ET. Cyclooxygenase and lipoxygenase inhibitors fail to block ET-induced contraction (1), demonstrating that $PGF_{2\alpha}$ and TxA_2 do not mediate the contractile activity. In fact, indomethacin strongly amplifies the pressor action of ET, indicating that a vasodilatory prostaglandin (probably PGI_2) attenuates the pressor action of ET (56). A modest depressor phase usually precedes the pressor action of ET, but this effect is insensitive to indomethacin, which suggests that EDRF mediates the initial depressor activity of ET (56). Thus analagous to the action of other vasoconstrictor peptides, PGI_2 might work in concert with endothelium-derived relaxing factor to dampen vasoconstriction by ET and form a negative feedback loop. Prostaglandin metabolites did not mediate or inhibit contraction of mesangial cells (58); however, ET-1 stimulation of PGE_2 amplified cAMP accumulation in response to β-adrenergic agonists (58). Another possible role for ET-stimulated PLA_2 is hormone-mediated lipid regulation of guanylate cyclase activity, which has been proposed to regulate intracellular cGMP levels in many tissues by increasing the cytosolic concentration of arachidonic acid, lysophospholipids, or fatty acid hydroperoxides (see ref 60). cGMP produced in this manner could constitute a negative feedback system to dampen vasoconstriction by ET. Much work is necessary to determine whether prostaglandin metabolites mediate ET actions or whether prostaglandins act mainly as negative feedback signals.

ET and the adenylate and guanylate cyclase cascades

Several reports suggest that ET fails to activate adenylate or guanylate cyclase. In cultured vascular smooth muscle (61) and mesangial cells (58), ET did not alter basal levels of cAMP. ET actually caused a modest decrease in basal cAMP levels in 3T3 fibroblasts (39). cAMP accumulation was similarly unaffected by application of ET to intact preparations of rabbit aorta (44) and rat atria (40). cGMP levels were unchanged when ET was added to endothelium-intact or endothelium-denuded aortic rings (44). These studies should be considered preliminary as only ET-1 was used, and other tissues need to be tested; however, current evidence argues against direct activation of adenylate or guanylate cyclase by ET receptors.

Potential involvement of other ionic and biochemical signaling systems

Other signaling systems undoubtedly contribute to the biological actions of ET. One possible candidate is membrane potential, which helps determine the electrochemical driving force for many ions involved in signal transduction and can gate voltage-sensitive ion channels or other transport systems. ET causes sustained, dose-dependent depolarization of membrane potential in vascular smooth muscle (34, 36) and ciliary muscle (62) cell lines. In one study, depolarization was immediately preceded by transient hyperpolarization due to activation of a Ca^{2+}-sensitive K^+ channel by ET (34). These investigators concluded that ET depolarizes membrane potential by gating a Ca^{2+}-permeable, nonspecific cation channel, and that the resulting depolarization gates an L-type VOC Ca^{2+} channel. Gating of a similar nonspecific cation channel and subsequent depolarization have been shown to occur after addition of arginine vasopressin to A7r5 vascular smooth muscle cells (63). In addition, data from our laboratory employing fluorimetric techniques to measure transmembrane cation permeability are also consistent with gating of a Ca^{2+}-permeable, nonspecific cation channel by ET in mesangial cells (see above, and ref 49). Thus sustained depolarization of membrane potential caused by gating a nonselective cation channel could be a common signal after activation of ET receptors and would have especially important consequences in electrically excitable cells.

The role of Na^+ conductance in ET-induced signal transduction is currently under investigation. Although ET is homologous to scorpion α-toxins (1), which activated VOC Na^+ channels, there is little evidence that depolarization by Na^+ influx contributes to ET-induced contraction (64). It has been shown, however, that high concentrations of ET inhibit Na^+-K^+-ATPase in epithelial (65) and vascular smooth muscle cells (66). The resultant increase in $[Na^+]_i$ has been suggested to reduce activity of the Na^+-Ca^{2+} exchanger, thereby reinforcing the sustained increase in $[Ca^{2+}]_i$ after addition of ET (66), but this hypothesis remains to be tested.

We and others have shown that ET strongly activates amiloride-inhibitable Na^+-H^+ exchange, causing marked alterations of pH_i (41, 42, 66). In mesangial cells loaded with the pH-sensitive probe, BCECF, concentrations of ET-1 that increase phospholipase C activity also increase Na^+-H^+ antiport (7, 41). ET-1 causes a dose-dependent, net alkalinization of 0.1–0.3 pH units. As reported for agonist-induced activation of Na^+-H^+ in other cell types, ET-induced alkalinization is often preceded by a short, transient acidification that most likely relates to the increase in $[Ca^{2+}]_i$ (67). Although the precise role for Na^+-H^+ exchange (or changes in pH_i) in signal transduction remains to be clarified, it seems possible that under certain conditions, ET-stimulated Na^+-H^+ exchange might contribute to the promitogenic action of ET (see ref 67 for review).

SIGNAL TRANSDUCTION IN THE NUCLEUS BY ET

Our discussion has focused on short-term pathways of signal transduction that potentially mediate rapid ET-induced biological actions such as contraction and secretion. But the finding that ET is a potent mitogen for cultured cells (38, 39, 41, 42, 68, 69) demonstrates that ET differentially regulates gene expression to produce long-lasting responses as well. An understanding of the pathways by which ET activates genes will be critical to elucidate the physiological and pathophysiological roles of ET. The following discussion summarizes what little is known about how ET peptides regulate gene expression by activating DNA binding transcription factors for RNA polymerase II.

ET activates transcription of the *c-fos* proto-oncogene

c-Fos is the prototype for a group of inducible genes that convert short-term, transmembrane signals into long-term responses requiring transcriptional regulation of target genes (see refs 70, 71 for review). Thus *fos* genes act as switches for activating transcription of gene networks in response to a variety of extracellular signals such as growth factors or neurotransmitters. The *fos* proto-oncogene (*c-fos*) is the normal counterpart of the transforming oncogene (*v-fos*) encoded by the FBJ and FBR murine osteogenic sarcoma virus (see ref 70). The *c-fos* gene belongs to a heterogeneous family of the transcription factors encoded by multiple genes. Through formation of leucine zippers, *fos* proteins form heterodimers with other related members of the Ap-1 family (72); these heterodimers then bind to consensus sequences to regulate transcription (72, 73). Expression of the *c-fos* gene occurs rapidly after the addition of ET-1 to glomerular mesangial cells (41), vascular smooth muscle cells (68, 69), and 3T3 fibroblasts (39). ET causes greater induction of the *c-fos* gene in mesangial cells than did 5% FBS, a potent stimulus of *fos* induction (41). Expression of *fos* is maximal at 30–60 min and is undetectable by 120 min. Such rapid and transient kinetics are hallmarks of *c-fos* gene induction in other cells stimulated by many agonists (70, 71). Thus ET appears to activate *fos* gene transcription, but the consequences of ET-induced *c-fos* induction on target gene transcription have yet to be investigated.

How does ET activate transcription of the *c-fos* gene?

Previous work demonstrates that *c-fos* induction can be triggered via three pathways. Activation of *c-fos* transcription by serum or growth factors requires an SRE located 300 bp upstream of the transcriptional start site (74). Induction of the *c-fos* gene via SRE occurs by at least two independent signal transduction pathways, one involving protein kinase C and the other independent of protein kinase C. Another pathway for induction of *c-fos* requires a CRE and the CRE-binding protein (70, 71). This pathway is activated by agents that increase adenylate cyclase activity (75). In some systems an increase in $[Ca^{2+}]_i$ increases *c-fos* transcription, and both the $[Ca^{2+}]_i$- and cAMP-sensitive pathway are independent of SRE. Thus *c-fos* transcription can be activated by multiple independent signal transduction pathways. As discussed above, there is little evidence that ET activates adenylate cyclase, and therefore it is unlikely to increase *c-fos* transcription by a CRE-sensitive pathway. By activating the phosphoinositide cascade, ET increases both protein kinase C activity and $[Ca^{2+}]_i$. It is plausible to speculate that ET activates *c-fos* transcription via the SRE- or Ca^{2+}-sensitive pathways.

Multiple nuclear signaling pathways are, in part, responsible for differences in biological actions evoked by different hormones that cause similar induction of *c-fos* (70, 73). ET activates genes for other transcription factors that either act independently or interact with *c-fos* to regulate transcription (see **Fig. 6**). Experiments suggest that ET also increases transcription of *c-myc* (68) and VL30 genes (38). Increased expression of VL30 gene products by ET is especially interesting as the VL30 elements are a family of mouse genes resembling integrated proviral forms of retroviruses, and these genes are activated by other promitogenic agents including epidermal growth factor and phorbol ester (76). These data suggest that activation of the phosphoinositide cascade, and other as yet undefined signals, mediate nuclear signaling by ET and are responsible for long-lasting biological changes induced by ET peptides.

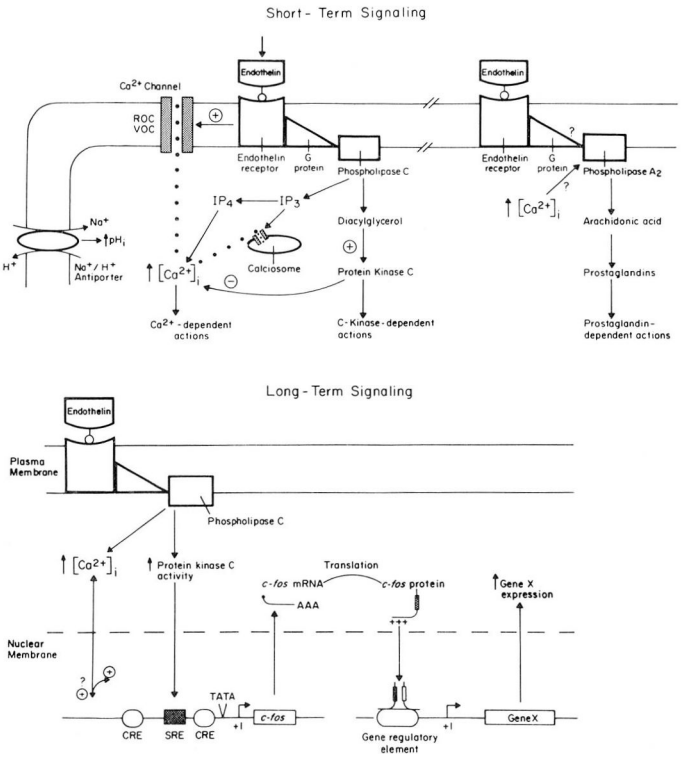


Figure 6. Short-term and long-term models of cellular signaling by ET peptides in target cells. The salient features of this model are discussed in the text.

ET: A MODEL FOR CELLULAR SIGNALING

The preceeding sections reviewed the available evidence for activation of signal transduction pathways by ET. A tentative model for cellular signaling by ET is summarized schematically in Fig. 6. This model emphasizes temporal differences in signaling and suggests a possible mechanism whereby short-term responses might be amplified to regulate long-term changes in gene expression.

1) ET elaborated by specific endothelial and epithelial cells acts in a paracrine fashion on nearby smooth muscle cells, fibroblasts, or related cells such as retinal pericytes or glomerular mesangial cells.

2) ET binds to target cells on cognate receptors (or receptor subclasses) distinct from DHP-sensitive Ca^{2+} channels. The ET-receptor complex activates phospholipase C through interaction with a putative G protein, perhaps constituting the initial transmembrane event.

3) The phosphoinositide cascade produces two second messengers: $Ins(1,4,5)P_3$ and diacylglycerol. The water-soluble $Ins(1,4,5)P_3$ diffuses into the cytoplasm and releases Ca^{2+} from the intracellular stores, thereby increasing $[Ca^{2+}]_i$. Ca^{2+} signaling undoubtedly mediates many, but not all, ET-induced biological actions. $Ins(1,3,4,5)P_4$ might function as an additional Ca^{2+}-mobilizing second messenger. The neutral diacylglycerol remains within the plasma membrane and activates protein kinase C, which contributes to ET-induced biological responses and triggers a negative feedback signal to dampen Ca^{2+} signaling by ET.

4) ET gates multiple types of Ca^{2+} channels, possibly in a cell-specific manner, but the mechanisms by which ET increases Ca^{2+} influx remain poorly characterized. Ca^{2+} influx via these channels produces a sustained increase in $[Ca^{2+}]_i$. Through an indirect, modulatory mechanism, ET increases the probability of L-type Ca^{2+} channels to open during depolarization.

5) ET also stimulates phospholipase A_2 to release arachidonic acid from membrane phospholipids. Free arachidonate is then converted to bioactive eicosanoids. ET might activate phospholipase A_2 via interaction with a G protein or by increasing $[Ca^{2+}]_i$, or both.

6) ET also causes other ionic and biochemical changes that contribute to cellular signaling. These changes include depolarization of membrane potential, activation of electroneutral Na^+-H^+ antiport, and inhibition of Na^+-K^+-ATPase.

7) ET also causes long-term, adaptive responses that require changes in gene expression. One possible mechanism is that short-term signals evoked by ET stimulate transcription of the *c-fos* gene, which in turn activates transcription of gene networks to cause adaptive changes. As shown in Fig. 6, both the increase in $[Ca^{2+}]_i$ and protein kinase C activity could promote transcription of the *c-fos* gene. Through formation of leucine zippers (72), newly synthesized *c-fos* protein forms heterodimers with other related *trans*-acting factors. These heterodimers then bind to *cis*-acting sequences on target genes to regulate transcription. Multiple pathways undoubtedly mediate nuclear signaling by ET.

CONCLUDING REMARKS

We have summarized the pathways of signal transduction after binding of ET to receptors on target cells. Here we have focused on short-term signaling and have only briefly examined the important question of how transmembrane signals flow to the nucleus, causing differential regulation of gene expression and long-term effects. Although our knowledge of many components is incomplete, the outline of ET-activated systems of signal transduction are clear and bear striking resemblance to phosphoinositide-linked signaling systems activated by other regulatory peptides. It is also clear that the ability of cells to respond to ET is tightly regulated and that significant differences exist in the signaling pathways responding to ET in different cell types. Further progress in understanding the physiological and pathophysiological roles of ET peptides will depend on exploring in detail the mechanisms by which ET elicits complex biological events. FJ

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