THE ROLE OF POTENTIALLY PHOSPHORYLATED SERINES IN DESENSITIZATION OF THE ENDOTHELIN-A RECEPTOR

by

Jay S. Rosenbloom

A DISSERTATION

Presented to the Department of Cell Biology and Anatomy
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

September, 1994

APPROVED:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

TABLE OF CONTENTS

DEDICATION

This thesis is dedicated to my parents and my grandparents. Each of whom was committed to sacrificing whatever was necessary to provide their children with more opportunities then they themselves enjoyed. This thesis is also dedicated to my wife, Michelle Mendelson Rosenbloom, without whom this thesis and my life in general would be of lesser quality.

ACKNOWLEDGMENTS

I thank Dr. David Pribnow for having had the confidence in me to take me into his laboratory, and for teaching me all about how to be a scientist. The knowledge and experience I gained while working with him will be useful not only in scientific persuit, but throughout life in general. I also want to thank Dr. Pribnow for having given me the freedom to learn and experiment along the way without letting me get to far afield of my goals. Working together at every stage of my project proved to be a gratifying and rewarding process for me.

I thank Dr. Caroline Enns for her help along the way. She helped me to understand what I was doing and why I was doing it. I thank Dr. John Adelman and Dr. James Maylie for their time and resources in teaching me, and helping me investigate the world of oocyte electrophysiology. I thank Dr. Paula Stenberg for her warmth and generosity in attempting to look at my receptor through the electron microscope. I thank Dr. Bruce Magun for providing me with the supplies and space necessary to undertake this research project. I thank Dr. Deborah Weiss, Angela Horgan and Michelle Rosenbloom for their literary assistance, and for having persisted through drafts of documents that were not fit for human consumption. I would like to thank all of the people in the lab and throughout the department who made my tenure in the laboratory bearable, and sometimes even fun. And finally I would like to thank my mother, my father, my sisters Sheila and Debra, and my wife Michelle for having given me the love and support through good times and bad, and for having made all of this possible. Thank you all.

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Human endothelin-A receptors (ET_AR) are desensitized when exposed to endothelin-1. Desensitization is a two part process including decrease in signaling and internalization of receptors. However, the molecular mechanisms underlying these processes are not well characterized for the endothelin receptors. Phosphorylation of serine residues in the third intracellular loop and carboxyl tail of several G-protein coupled receptors are known to be involved in agonist-induced desensitization. Therefore, the potential role of phosphorylation of ET_AR serines in agonist-induced desensitization was investigated. Ser-289 in the third intracellular loop and Ser-391, Ser-393, Ser-420 and Ser-421 on the carboxyl tail of the ET_AR were replaced with alanines by site-directed mutagenesis. Mutant and non-mutant constructs were expressed in Xenopus oocytes and in stably transfected CHO-K1 cells. No significant differences were found in desensitization between the mutant and non-mutant receptor constructs in either of the expression systems. These results suggest that phosphorylation of the five selected serines is not involved in desensitization of the endothelin-A receptor.

CHAPTER 1
INTRODUCTION

Arteries, veins and capillaries are the conduits that deliver blood to peripheral tissues. Neural and hormonal signals regulate the tone of these vessels. Coordination of complex signals results in local and systemic regulation of blood pressure and blood flow. Vascular tone is the result of a net difference between relaxation and contraction. Endothelial cells that line blood vessels affect the tone of underlying vascular smooth muscle through the production of several paracrine factors (1). For example, the transmitter acetylcholine stimulates vasculature relaxation indirectly through endothelial cells (1). Relaxation factors derived from endothelial cells include prostacyclin and nitric oxide (2, 3). Epinephrine, arachidonic acid, physical stretching and increased transmural pressure have been found to cause endothelium dependent vasoconstriction (4). A protease-sensitive vasoconstrictor was described in the supernatant fraction of bovine endothelial cells prior to the purification of the factor (5-7). The exact nature of the agent responsible for this vasoconstriction was unknown until 1988 when Yanagisawa reported the isolation of a novel vasoconstrictor, endothelin (8).

PRODUCTION OF ENDOTHELINS

Endothelin (ET) is a peptide vasopressor purified from the conditioned media of porcine aortic endothelial cells. The mature form is 21 amino acids in length, and contains two disulfide bonds yielding a bicyclic structure (8). The porcine hormone is synthesized as a 203 amino acid preproendothelin (see figure 1). The first 17 amino acids represent the signal peptide required for the protein to enter the secretory pathway (9). Paired basic amino acid residues Lys51-Arg52 and Arg92-Arg93 are recognized and cleaved by endopeptidases, resulting in the 39 amino acid intermediate 'big-endothelin' (big ET) (8). The previously unknown endothelin converting enzyme (ECE) is

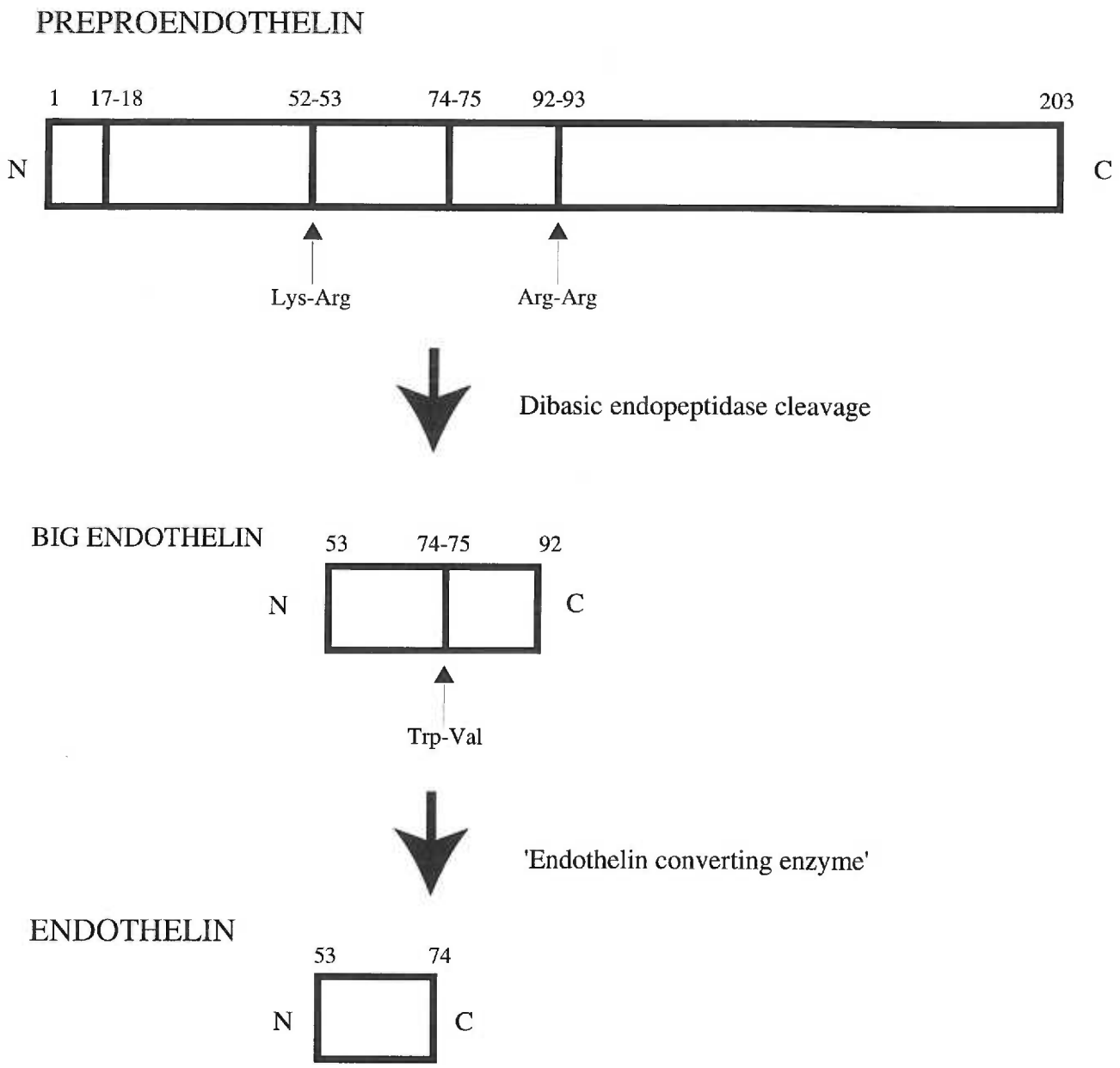


Figure 1. Biosynthetic processing pathway for endothelin. The secretory signal sequence represents amino acids 1 to 17. Residues 18 to 52 and 93 to 203 are cleaved by a dibasic pair specific endopeptidase. The final cleavage to form mature endothelin is by an endothelin converting enzyme, and removes amino acids 75 through 92.

responsible for the final processing of mature endothelin, which is generated through an unusual proteolytic cleavage between Trp74-Val75 (8). Vasoconstrictor activity of ET is about 140 times higher than big-ET on a molar basis (10). ET induces a 50% maximal response at doses more than an order of magnitude lower than values reported for angiotensin II, vasopressin, or neuropeptide Y, making ET the most potent mammalian vasoconstrictor peptide known (8). Research is being directed at cloning ECE so that selective antagonists can be identified. The enzyme has been partially purified from human endothelial cell hybrids, and is known to be an integral membrane protein (11). Pharmacologic inhibition of the converting enzyme is predicted to parallel the effects of angiotensin converting enzyme (ACE) inhibitors, which have been extremely successful at treating hypertension.

ENDOTHELIN ACTION

Endothelin's role in normal physiology has been difficult to elucidate. One difficulty is a result of the potency of ET, and the fact that its effects are likely paracrine in nature. Plasma levels of ET measured in normal human controls were 1.5-2 pg/ml, which is three orders of magnitude lower than the 50% effective dose for inducing vasoconstriction, *in vitro* (12). The discrepancy in hormone levels may be due to variability in assay techniques, but is more likely the result of tissue ET overflowing into blood. Another complicating factor is that ET is secreted in response to a wide range of stimuli. For example, agents that stimulate the release of endothelin from vascular smooth muscle cells include vasopressin and angiotensin II (13). Moreover, ET stimulates the secretion of an equally long list of compounds including thromboxanes, prostaglandins, progesterone, epinephrine and others (13). ET has also been shown to stimulate secretion of neuroendocrine hormones from anterior pituitary and adrenal glomerulosa cells (14, 15).

Functional responses to endothelin include constriction of a variety of smooth muscle targets including airway, uterus and vas deferens (13). Endothelin acts as a potent mitogen for fibroblasts and vascular smooth muscle cells, and induces hypertrophy in cardiomyocytes (16-18). Endothelin's most striking effect is the increase in blood pressure it produces *in vivo*. Intravenous administration of a single dose causes a transient decrease in arterial pressure, followed by a slowly developing elevation (8). This increase in tension is sustained for 40-60 minutes, while the plasma half-life of the hormone is a mere 40 seconds (8, 19). Preliminary *in vivo* studies have been done in humans investigating the effects of ET on regional blood flow. Administration of a single dose elevated the arterial pressure for more than one hour in splanchnic vessels and more than three hours in renal vessels (20).

ENDOTHELIN ISOFORMS

Three isoforms of endothelin have been identified (21). Each of the peptides is structurally and pharmacologically distinct, and each is coded for by a separate gene. The genes encoding the three endothelins were cloned by screening a human genomic DNA library under low stringency hybridization (21). Endothelin-1, -2, and -3 (ET-1, ET-2, & ET-3) share a high degree of sequence homology and are similar in secondary structure (see figure 2). The only differences between ET-1 and ET-2 are amino acids six and seven. ET-3 has less sequence homology, six of its 21 amino acids differ from ET-1. Sarafotoxins (STRX), a family of peptides strikingly similar to ET are found in the venom of the Israeli snake *Atractaspis engaddensis* (for one example see figure 2) (12). A bite from this species of snake can be lethal, this is thought to be the result of constriction of the coronary arteries and a blockage of the A-V node. To date, four sarafotoxins have been characterized (SRTX-a, -b, -c and -d). They are structurally and functionally similar to the endothelin peptides, and have been useful tools in studying the effects of endothelins (4).

TISSUE DISTRIBUTION

The three isoforms of ET are coded for by separate genes, regulated independently, and are expressed in different proportions in various tissues (21, 22). The isoforms of the hormone have overlapping tissue distributions, but the potency of their biological effects varies widely between tissues (21). Distribution has been assayed by several techniques with varying sensitivities in cultured cells, tissue samples and whole animals. Northern blot analysis revealed that ET-1 is the only isoform expressed in vascular endothelial cells (4, 23, 24). Radioimmunoassay measurements of tissue samples found ET-1 in the kidney, lung, heart, intestine, pancreas, pituitary, neurons of the spinal cord and dorsal root ganglia, and other tissues (19, 25, 26). Radioimmunoassay also detected ET-1 in blood, cerebrospinal fluid and urine (24).

The expression of ET-2 mRNA is restricted to the intestine in normal tissue, but has also been detected in a kidney tumor cell line (19, 26, 27). Monkey Cos-7 kidney cells also produce ET-2 (24). ET-3 mRNA has been detected in intestine, lung, pituitary, pancreas and kidney tissues (23, 26). Radioimmunoassay detected ET-3 in pituitary, kidney, intestine, lung and brain tissues (19, 24).

ENDOTHELIN IN DISEASE

Endothelin's wide ranging effects on a variety of biological systems has generated a great deal of interest in its possible role in disease. Studies have reported significant elevation in plasma endothelin levels associated with a variety of pathologic conditions. Elevated plasma endothelin levels have been reported in cardiovascular pathophysiology including cardiogenic shock, acute myocardial infarction, hypertension, idiopathic cardiomyopathy, congestive heart failure and arteriosclerosis (12, 19, 28). Elevations in circulating ET may reflect increased production, death of ET producing cells, or an impairment in the clearance of ET from plasma. Increased circulating endothelin will induce vasoconstriction, reduce regional blood flow and stimulate mitogenesis of smooth

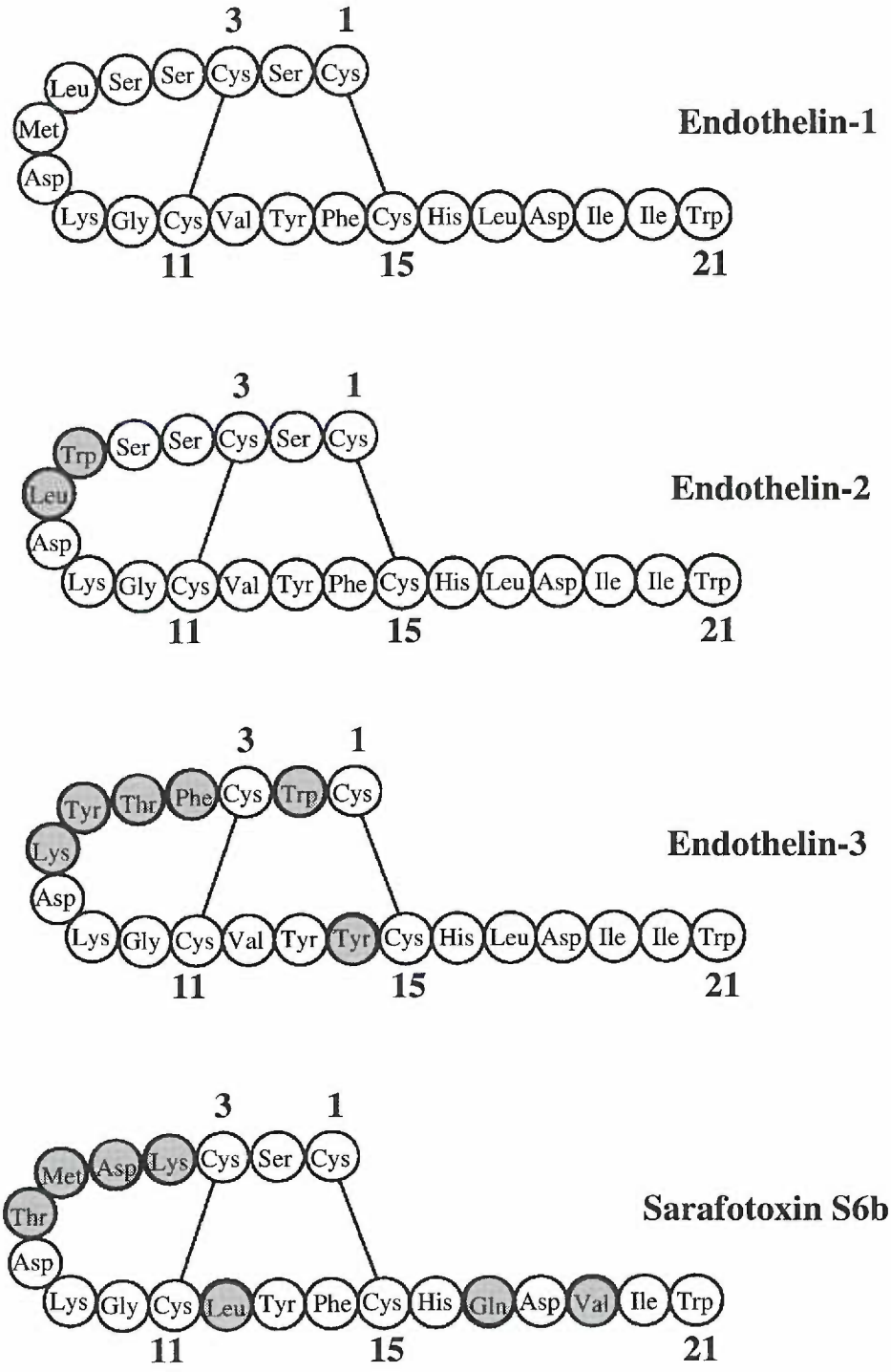


Figure 2. Amino acid sequences of mature endothelin-1, endothelin-2, endothelin-3 and the snake venom sarafotoxin S6b. Numbering starting at 1 represents amino terminus, and number 21 represents carboxy terminus. The line from amino acids 1 to 15 and 3 to 11 represent disulfide bonds.

muscle cells. It will also cause cellular hypertrophy, increasing the mass of the cells without increasing their numbers (29). A recent study of patients suffering myocardial infarctions reported that increased levels of endothelin in plasma gave a more accurate prognosis of one-year mortality than age, sex, previous medical history, or in-hospital treatment (30). Other conditions in which elevated endothelin levels have been reported include systemic lupus erythematosus, inflammatory arthritis, uremia, sepsis, Raynaud's disease, surgical operations, trauma, renal occlusion and others (13, 19). It has been repeatedly reported that cellular injury or death occurs when cells that have been deprived of blood are suddenly reperfused. The injury resulting from the return of blood flow is called reperfusion injury (30). One specific result of reperfusion that has been noted is the constriction of blood vessels (29). This decrease in blood flow supplying post-ischemic tissue could potentially result in increased cellular damage. To test ET-1's role in reperfusion vasoconstriction, animals received a subarachnoid hemorrhage, or mechanical occlusion of arteries to the kidney or liver. Prior to reperfusion, an orally active endothelin receptor antagonist or antisera against ET-1 was administered to the animal. All of the organ systems tested revealed that inhibition of endothelin's action greatly ameliorated the vasoconstriction associated with post-ischemic injury (29, 31, 32).

Abnormal levels of endothelin receptor expression may contribute to pathophysiology. Endothelin receptors are upregulated in heart tissue following ischemia, in kidney tissue following endotoxin exposure or cyclosporin administration, and in the bladder and prostate of patients with benign prostatic hypertrophy (33-35). These studies suggest that endothelin and its receptor may be involved in the pathogenesis of a variety of disease states.

ENDOTHELIN RECEPTORS

Prior to the cloning of any endothelin receptors there was evidence to suggest the existence of multiple subtypes. Kloog proposed a three receptor type classification based

on differences in agonist affinities revealed by binding studies (36). The classification predicted one type that preferentially binds ET-1, one type that binds all three peptides equally well, and one type that preferentially binds ET-3. To date, two receptor subtypes have been cloned from mammalian sources, and a third receptor type has been cloned from a non-mammalian source.

ET_AR

The first subtype is the endothelin-A receptor (ET_AR). The receptor was originally cloned from bovine sources using an electrophysiologic assay of Xenopus oocytes expressing exogenous mRNAs (37). It has since been cloned and characterized from human tissue (38-41). Competition studies revealed that the relative binding affinities of ET-1, ET-2 and ET-3 to ET_AR are about 1:7:900, respectively (8, 42). Tissue distribution studies of ET_AR have been variable in their results. Discrepancies are probably due to technical differences in the methods used. Tissue localization studies have utilized Northern hybridization, pharmacological binding studies with ^{125}I ET-1 vs. ^{125}I ET-3, and *in situ* hybridization. The cumulative data suggest that ET_AR has highest expression levels in vascular smooth muscle, heart, lung, brain, adrenal, liver, gut, kidney, uterus, placenta and testis (13).

ET_BR

The second type of endothelin receptor, the endothelin-B receptor (ET_BR), was originally cloned from rat tissue (43). This receptor has since been cloned from human tissue, and is 55% identical to the human ET_AR (44). Competition binding revealed that all three isoforms of ET share equal affinity for ET_BR (44-46). The expression of this non-selective receptor has been reported in endothelium, heart, lung, brain, adrenal, liver, gut, kidney, uterus, aorta and placenta (see(13) for review).

ET_CR

An ET_CR is predicted based on pharmacological studies of ET binding to mammalian tissues (36). The only source from which a clone has been derived is melanophores of the non-mammalian vertebrate *Xenopus laevis* (47). The clone was isolated through a combination of techniques, including polymerase chain reaction with degenerate primers, and subsequent screening of a cDNA library. The deduced amino acid sequence is 47% and 52% identical to ET_AR and ET_BR respectively (47). Functional studies of the ET_CR expressed in melanophores revealed that it responds to ET-3 about 200 times more effectively than ET-1 or ET-2 (47). Although this receptor has not been cloned from mammalian sources, it has been reported that in some bovine pituitary and endothelial cells ^{125}I ET-3 has a higher affinity than ET-1, and ET-3 has stronger biological activity than ET-1 (48).

ETR SIGNALING

Activation of endothelin receptors is the result of a ligand binding event, which triggers a variety of cellular signaling events. Some variability of resulting signals in different cell types is the result of co-expression of different ratios of ET_AR and ET_BR (13). It is also likely that different cell types expressing the same receptor signal through divergent pathways. The common element in the various down stream signals is G-protein coupling. Most cells that express the A isoform of the endothelin receptor activate the G-proteins that activates phospholipase Cβ (PLC) (12). The activated PLC then hydrolyses phosphatidylinositol, producing diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3). DAG subsequently activates protein kinase C (PKC), and IP_3 activates its receptor stimulating the release of intracellular stores of calcium (49). Activated PKC and increased intracellular calcium can have a variety of effects ranging from increasing DNA synthesis to mechanical contraction of the cell (50).

Other signal transduction pathways which respond to endothelin via the ETRs include phospholipase D (PLD), phospholipase A2, ion channels, activation of adenylyl cyclase, and inhibition of adenylyl cyclase (12, 13). Studies are ongoing to determine which endothelin receptor subtype is responsible for each signaling mechanism.

G-PROTEIN COUPLED RECEPTORS

ET_AR, ET_BR, and ET_CR have in common the structural and functional properties characteristic of the most prevalent and diverse of all cell surface receptors, the G-protein coupled receptor superfamily (GPCR) (51). All family members contain seven hydrophobic domains which alternate with hydrophilic domains. The hydrophobic domains span the plasma membrane, and the hydrophilic domains form the intracellular and extracellular loops. The result is a family of receptors with an extracellular amino terminus, three intracellular loops alternating with three extracellular loops and an intracellular carboxyl tail.

Diversity within the family of GPCRs is emphasized by the wide variety of associated ligands. Included are amine receptors such as the α and β adrenergic receptors, as well as dopaminergic, histaminergic, muscarinic and serotonergic receptors (see (52) for review). Many GPCRs are peptide receptors, including the receptors for angiotensin, bombesin, thrombin, tachykinin, follicle stimulating hormone and luteinizing hormone. Several GPCRs are involved in sensory stimuli, such as odorant receptors, bitter and sweet taste stimulants, and visual rhodopsin receptors (52, 53). Data bases of GPCRs include more than 100 distinct receptor types, and this list is continually expanding (54, 55).

GPCRs are so named because activation of guanine nucleotide binding (G) proteins is an intermediate stage in their signal transduction. G-proteins consist of heterotrimeric complexes of distinct alpha, beta and gamma subunits. Multiple isoforms of each subunit contribute to the diversity in signaling via the GPCR pathway (56, 57).

When agonist binds to a GPCR, the conformation of the receptor changes enabling interaction with a G-protein. The inactive G-protein then exchanges bound GDP for intracellular GTP. The result of this substitution is an active G-alpha dissociating from G-beta/gamma. The G-beta and G-gamma do not dissociate from one another (57). Active G-alpha activates a variety of effectors including adenylyl cyclase, Ca^{2+} and K^+ channels, phospholipase A_2 (PLA_2), PLC, phospholipase D (PLD), and cGMP phosphodiesterase (52, 58). At one time, G-protein function was fully attributed to the alpha subunit, while beta and gamma were only thought to be involved in anchoring the complex to the plasma membrane (54). Beta-gamma complexes are now known to effect adenylyl cyclase, PLC, PLA2, cGMP phosphodiesterase, K^+ channels, as well as different forms of G-protein receptor kinases (57). One example of the differential activation has been seen with different subtypes of PLCβ. $G_{\beta\gamma}$ subunits stimulate PLCβ3 most strongly and PLCβ4 not at all, whereas $G_{\alpha q/11}$ subunits stimulate PLCβ1 most and does stimulate PLCβ4 (58).

RECEPTOR DESENSITIZATION

Specialized cellular systems have evolved to coordinate complex signaling processes. These systems contain regulatory loops that feed back and regulate activity at several levels, particularly at the receptor level. Activation of a specific receptor stimulates regulatory systems, resulting in a dampening of the signal that initiated the loop. Reduction of signaling, or desensitization, is found in the presence of continuous or repeated stimuli. Different types of desensitization can be separated out as either receptor-specific (homologous) or generalized (heterologous). Homologous desensitization serves to reduce the cellular response to a distinct receptor stimulus. Heterologous desensitization describes a reduction in responsiveness of receptors unique from those involved in the initial signaling event.

DESENSITIZATION MODEL

Many of the mechanisms involved in desensitization were first discovered and characterized in the β-adrenergic receptor (βAR), which is a member of the GPCR family. The characteristics identified in the βAR system have been identified in many of other GPCRs. One model developed for βAR desensitization predicts a three stage process: uncoupling, internalization and down regulation (59).

UNCOUPLING/PHOSPHORYLATION

Uncoupling occurs in the seconds following receptor activation. This event causes signaling to diminish, while the receptor remains in the plasma membrane. Uncoupling represents the functional separation of the ligand-bound receptor from the signaling G-protein. In many of the GPCRs studied, uncoupling is mediated by phosphorylation. A list of GPCRs that are known to be phosphorylated in the process of desensitization can be found in table 1.

GPCRs are phosphorylated by two types of cellular kinases. One type, effector kinases, includes protein kinase-A (PKA) and PKC. PKA is activated by cAMP, and PKC is activated by DAG. These kinases provide a potential mechanism for wide cellular effects because their targets are not receptor specific.

The other cellular kinase involved in phosphorylation of GPCRs is the G-protein coupled receptor kinase (GRK). These kinases are unique in that they phosphorylate GPCRs only when they are occupied by ligand (60). Members of this kinase family include rhodopsin receptor kinase (GRK1), two β-adrenergic receptor kinases (GRK2 and GRK3, also known as βARKs), a gene cloned from the Huntington's disease locus (GRK4), and recently GRK5, GRK6, and GRK7 were identified in neutrophils (60, 61). *In vitro* phosphorylation of GPCRs by these kinases reduces, but does not inhibit,

Receptor	Source of receptor	Reference
α-mating factor receptor	yeast	(62)
Muscarinic cholinergic receptors	human	(63)
Rhodopsin receptor	bovine & frog	(64)
Thrombin receptor	human	(65)
β adrenergic receptor	human	(59)
α adrenergic	murine	(66)
Formyl peptide receptor (fMLP)	human	(67)
C5a chemoattractant receptor	human	(67)
Olfactory receptors	rat	(68)
Follitropin (FSH)	rat	(69)
Substance P receptor	rat	(70)

Table 1. G-protein coupled receptors phosphorylated in the process of desensitization.

receptor activation of G-proteins (71). Phosphorylation of receptors by GRKs potentiates binding of an arrestin protein. Once an arrestin binds to the receptor, the conformation of the complex changes so that signaling can no longer occur.

Binding of β-arrestin proteins is required to fully quench the signaling of β-adrenergic receptors (72). Three mammalian genes have been cloned for different forms of arrestins, all are expressed as splice variants (73). The first arrestin was discovered to be the inhibitor of visual signaling triggered by light-activated rhodopsin. The other two arrestins, β-arrestin 1 and β-arrestin 2 , were discovered through their effect on the β-

adrenergic receptor (71). Receptor-arrestin complexes are stable until the ligand has left the receptor, at which time, arrestin is released and the receptor becomes sensitive to phosphatases (71). Removal of phosphates by phosphatase enzymes regenerates the receptor back to an inactive state, ready to be stimulated by ligand again.

INTERNALIZATION

Internalization is the second stage of desensitization. It is distinct from uncoupling of receptors and G-proteins. Receptors transform into an active conformation after binding ligand, and then internalize. Internalization involves sequestration of the receptor away from the cell surface into membrane bound vesicles (74), which Internalization occurs in the seconds to minutes following receptor activation (59). This is a well-described phenomenon characteristic of many GPCRs (for a list see table 2.). The consensus sequence of amino acids responsible for targeting GPCRs for internalization is not yet known (if it exists). It is known, however, that serines and threonines in the muscarinic cholinergic receptors and the gastrin-releasing peptide receptor, mediate internalization (75, 76). A recent report also suggests that a highly conserved tyrosine is required for agonist induced β2-adrenergic receptor internalization (77).

Clathrin coated pits (CCPs) within the plasma membrane bud off vesicles that form intracellular transport compartments which internalize cell surface receptors and their ligands. Different types of receptors are internalized via CCPs. These include receptors for protein hormones like EGF, receptors for transport proteins like transferrin, and even GPCRs like the β-AR (74, 78). Electron microscopy has been used to localize GPCR's route of internalization to CCPs (79). The most widely accepted model of internalization involves the CCP pathway, however, one report suggests that β-AR and yeast mating factor receptors are internalized via smooth, or non-clathrin coated pits (80).

RECEPTOR TYPE	REFERENCE
Bombesin receptors	(81)
Angiotensin II receptor	(82)
Neurotensin receptor	(83)
Thrombin receptor	(84)
Vasopressin receptor	(85)
M1 muscarinic cholinergic receptor	(86)
C5a chemoattractant receptor	(87)
Thyrotropin-releasing hormone receptor	(88)
Bradykinin receptor	(89)

Table 2. G-protein coupled receptors internalized in the process of desensitization.

DOWN REGULATION

The third stage of desensitization, down regulation, occurs after hours of stimulation (59). This regulatory process decreases the total number of receptors, which results in a diminished responsiveness. Down regulation has been characterized most extensively for the β-AR. Data suggest that β-adrenergic receptors do not have to be phosphorylated or sequestered in order to be down regulated (90, 91). The decrease in total cellular complement of receptors could be accounted for by decreased synthesis, enhanced degradation of receptors, or a combination of both. Site-directed mutation of two tyrosines in the C-terminus of a βAR did not interfere with signaling or recycling, but greatly diminished down regulation (92). The separation of signaling and internalization from down regulation in these receptors implies that the processes regulating down

regulation are at the level of the receptor protein. Other evidence suggests the opposite, reporting a reduction in receptor number parallels a decrease in mRNA stability (93). Agonist-induced reduction of mRNA levels has been reported for several GPCRs, including β2-AR, M1 muscarinic receptors, thyroid stimulating hormone receptors and α_{1b}-adrenergic receptors (93-96). The role down regulation plays in desensitization is clearly a long term process that provides feedback inhibition on an activated receptor signaling system.

ENDOTHELIN RECEPTOR DESENSITIZATION

Endothelin receptor regulation has a potentially important role in the maintenance of homeostasis within the cardiovascular system. A great deal of research has been done to identify the role of ETRs in normal physiology as well as its potential role in disease. Endothelin's action as a potent vasoconstrictor has motivated investigation of the desensitization of ET_AR in a variety of systems.

ET_AR EXPRESSION IN *XENOPUS* OOCYTES

Prior to cloning, expression of the endothelin receptor was studied using *Xenopus* oocytes injected with mRNA from rat heart (97). The first report of endothelin-A receptor cloning utilized an electrophysiologic assay in Xenopus oocytes, following mRNA injection (37). Electrophysiologic studies of ET_AR activation revealed a current that reflects a calcium-dependent chloride channel (97). Further characterization of ET_AR's properties in oocytes revealed that the receptor is profoundly desensitized following a single pulse exposure to endothelin (98). Desensitization resulted in complete loss of responsiveness to stimulation by endothelin for over an hour. Partial sensitivity to endothelin was regained after a 1.5 to two hour recovery period (99). This long lasting desensitization period makes the ET_AR unique among GPCRs (100).

ET_AR EXPRESSION IN CELL CULTURE

Desensitization of the endothelin-A receptor has also been investigated in cell cultures and preparations of vascular rings and strips. Cell culture experiments have used IP production as a measure of receptor activity. Desensitization can be defined in culture as a diminished production of IPs in response to a second dose of ET relative to the primary dose. Endothelin receptor desensitization has been demonstrated in a variety of cell types including neurohybrid (101), C-6 neurons (102), cerebellar granular cells (103), pituitary gonadotrophs (104), hepatocytes (105), tracheal smooth muscle cells (106) and cardiomyocytes (107).

Internalization is aspect of desensitization that results in the number of potentially activated receptors is decreased. The internalization of ETRs has been studied in cell culture. The formation of an endothelin receptor-ligand complex induces the rapid transfer from cell surface to an internalized compartment (105, 108). The fraction of receptors internalized varies among cell types. Primary cultures of rat hepatocytes internalize up to 90% of their total ligand-bound cell surface receptors (105). Primary cultures of human vascular smooth muscle cells internalize a maximum of 70% (108). Many details regarding the molecular mechanism responsible for internalization are unknown.

To determine whether ET_AR is internalized via a CCP pathway inhibition studies were performed. Two techniques known to inhibit CCP internalization are cytosolic acidification and pretreatment of cells with the transglutaminase inhibitor dansylcadaverine (108). When human vascular smooth muscle cells were treated by either method, internalization of ET_AR was significantly inhibited (108). This indicates that the receptor is internalized via the clathrin coated pit pathway.

Internalization and down-regulation both result in a decrease in cell surface receptors. It is not yet clear whether internalization is a precursor to down-regulation.

Cells pretreated with ET show a time and temperature dependent reduction in the total number of cell surface receptors (Bmax), without any change in the receptor's affinity for ligand (dissociation constant, or Kd) (109, 110). This indicates that the mechanisms involved in desensitization and internalization do not affect the affinity of the receptor for ligand.

ETR IN BLOOD VESSELS

Blood vessel strips from a variety of anatomical sites in a number of species have been used to study the *in vitro* effects of endothelin. The characteristic response of these vascular strips to a primary dose is a slow onset of tension (maximum at 11-16 minutes), and a very slow recovery (60-66 minutes) (111). Up to six hours after a primary stimulation with ET, rechallenging resulted in a less than maximal contraction (111).

ENDOTHELIN RECEPTOR ANTAGONISTS

A variety of different endothelin receptor antagonists have been identified which will provide better insight into the effects of ETs. Using antagonists against specific subtypes of endothelin receptors makes it possible to assess the role each plays in normal physiology and in disease states. The most widely used antagonist of ET_AR is BQ123. This cyclic peptide was developed based on the natural antagonist activity of a fermentation product of *Streptomyces misakiensis* (see figure 3.) (112).

Other examples of ET_AR antagonists include the pseudo-tripeptide FR-139317, and the orally active chemical compound called Ro 46-2005 (13, 32). To better understand ETR's role in basal vascular regulation, *in vivo* administration of an ET_AR antagonist was performed. When cats were infused with FR-139317 for up to 20 minutes vascular tone was completely unaffected (113). When a submaximal dose ($1.6x10^{-10}$ mol kg^{-1} min^{-1}) of exogenous ET-1 was infused for 20 minutes a distinct and reproducible

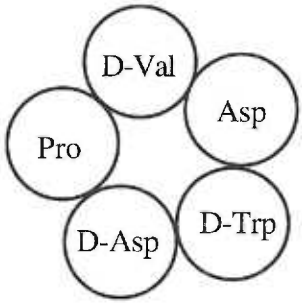


Figure 3. The structure of the endothelin-A receptor antagonist BQ123 (13).

vasoconstriction resulted. When both were administered together, FR-139317 inhibited the vasoconstrictor response to ET-1 in a dose-dependent manner (113). The effects of ET-1 and the ET_AR antagonist over the 20 minute period suggest that ET may be involved in long-term regulation, and perhaps during pathophysiological conditions.

Cardiac hypertrophy is a pathologic condition that is caused by a variety of events. One of the most serious causes of hypertrophy is myocardial infarction. Following an infarction, the heart is unable to pump sufficient volume of blood resulting in back pressure and hemodynamic overload. ET-1 has been shown *in vitro* to induce hypertrophy of cultured cardiomyocytes, and was thought to be a participant in post-infarction hypertrophy (114). A widely used model of hemodynamic overload involves banding the aorta in rats to restrict outflow from the left ventricle (115). Antagonizing the ET_AR by infusing BQ123 partially blocked the cardiac hypertrophy that resulted from pressure overload (115).

COMPARISON OF ET_AR TO OTHER GPCRS

Comparing ET-1 to other vasoconstrictors has suggested similarities in the signaling pathways and yet differences in the patterns of cellular effects. One indicator of

receptor signaling is intracellular Ca^{2+} levels. When aortic myocytes are stimulated with ET-1, angiotensin II (ANG II) or arginine vasopressin (AVP), the rate and duration of the rise in intracellular Ca^{2+} are equivalent (116). The tension induced in aortic rings as a result of stimulation by vasoconstrictors is quite a different story. The effects of ET-1 have slower onset and much longer duration. When ANG II and AVP are each applied to vascular strips both cause rapid increases in tension, which last about eight minutes (116). Endothelin, on the other hand, induces almost twice as much tension after only five and a half minutes, and maintains contraction for an hour (111, 116). Similarities in signaling with different end results suggests that ET_AR is very different from other vasoconstrictors acting through GPCRs.

MODEL OF ET_AR DESENSITIZATION

To better understand the role that endothelin receptors play in normal physiology and pathophysiology, it is important to determine how the receptor is regulated at the molecular level. To this end, I have embraced a model of desensitization based on literature describing the ET_AR specifically and more generally the G-protein coupled receptors.

I propose the following two step model for the desensitization of the ET_AR. The first step, phosphorylation of the receptor, occurs after hormone binding. When a receptor is occupied by ligand the conformation of the complex is changed, making the receptor a target for cellular kinases. The receptor is phosphorylated on serines located within consensus phosphorylation sites in the third cytoplasmic loop and carboxyl tail. Phosphorylation of the receptor would then interfere sterically or would induce a further change in receptor conformation, in either case preventing second messenger activation and subsequent ligand binding. The second step responsible for the ETR desensitization is internalization. Following ligand binding the cell is activated, the receptor is phosphorylated, and the receptor-ligand complex is endocytosed. Translocation from the

cell surface to cytoplasmic endosomes physically separates the receptors from available extracellular ligand. The net effect of these two steps is prevention of continual cellular activation in response to repeated or prolonged stimulation with ET.

This model of ET_AR desensitization is consistent with the general notion of GPCR studies indicating that phosphorylation of specific intracellular sites is involved in desensitization. Three authors reporting the deduced amino acid sequences of ET_AR have noted serines that may serve as substrates for cellular kinases. These serines are located within sequences that fit consensus motifs for PKC and CaM kinase II (117). The possibility of phosphorylation involvement has been tested using phorbol esters to mimic DAG and Ca^{2+} to activate PKC (49). When C-6 neurons, NG-108-15 cells, cardiomyocytes, tracheal smooth muscle cells or vascular smooth muscle cells are pretreated with phorbol esters the cellular responses to endothelin are diminished (101-103, 106, 107, 118). Pretreatment of cells expressing ET_AR with phorbol esters also results in a reduction in cell surface receptor numbers (102, 118). The results from these reports imply that PKC is involved in the desensitization of the ET_AR. Inhibition of PKC with the competitive antagonists staurosporine, sangiovamycin, or H-7 produced results that conflict with the phorbol ester data. Some investigators report these antagonists reverse the effects of the phorbol esters, while others report preincubation with PKC inhibitors had no effect on ET-1 induced desensitization (102, 106, 107, 109). The question of PKC involvement in ET_AR desensitization remains unanswered.

SUMMARY

The goal of this study was to gain insight into the regulation of endothelin receptor activity. This research was inspired by the notion that regulation of the response to a vasoconstrictor and mitogen plays a significant role in the maintenance of normal physiology. It can be anticipated that loss of this regulation would result in a pathophysiologic state. The experimental aims of this thesis were as follows:

1. Demonstrate desensitization of the cloned ET_AR.
2. Demonstrate that phosphorylation of ET_AR is involved in desensitization.
3. Demonstrate that phosphorylation of ET_AR mediates internalization which contributes to the desensitization process.

The detailed experiments, results and conclusions comprise the body of this thesis.

CHAPTER 2
MATERIALS AND METHODS

MATERIALS

^{3}H Myo-inositol, Expre$^{35}S^{35}S$ protein labeling mix, and ^{125}I ET-1 were purchased from NEN/DuPont. Endothelin-1 was from Peptides International. MCDB-302 media was from Sigma. pCDNA1neo plasmid was from Invitrogen. Then oligo-directed mutagenesis kit was from Amersham. Mouse 'M2' anti-flag IgG1 antibody and soluble flag peptide were from Kodak. Polyclonal rabbit anti-flag antibody #1968 was a generous gift of C. Enns. Goat anti-mouse IgG1 FITC was purchased from Boehringer Mannheim. Oligonucleotide primers were synthesized in our lab on a Cyclone oligonucleotide synthesizer.

PCR CLONING OF HUMAN ENDOTHELIN-A RECEPTOR

Total RNA was isolated from cadaveric human lung tissue by the acid phenol extraction process as described (119). Reverse transcription of RNA was primed with a combination of 10μg/ml oligo dT and 0.4μM oligonucleotide #153 (5' CAGTCTCGAGTCACAGTTGCCTTGTG 3') encoding the 3' untranslated region of the ET_AR and are located 52 to 71 base pairs down stream of the translational stop signal (homologous bases are underlined). The reverse transcription reaction was carried out by D. Pribnow. The resultant cDNA was used as the template in polymerase chain reactions (PCR) according to the technique described by Saiki et al. (121). The primers contained unique restriction digest sites 5' to the homologous priming sequences. Primers were as follows: 5' primer =5' CGTCAAGCTTCAAGATGGAAACCCTTTCG 3';
3' primer =5' CAGTCTCGAGGGTGGTCAGTTCATGCTGT 3' (nucleotides encoding the ET_AR are underlined). PCR synthesis included 1unit $Vent_R$ DNA polymerase (New

England Biolabs) in a 20µl reaction volume. Thermocycling involved a preliminary one minute denaturation at 95°C followed by 40 cycles of the following: 30 seconds at 95°C for denaturation, then 30 seconds at 45°C for annealing and finally one minute at 72°C of elongation. The product was subcloned into the HinD III and Xho I sites of the m13mp18 and pcDNA 1/neo vectors.

SITE DIRECTED MUTAGENESIS

Insertion of the flag epitope into the ET_AR cDNA was carried out using an oligonucleotide directed mutagenesis kit from Amersham corp. according to the procedural directions supplied by the company. For details about the primer used see figure 7. The first step in the insertion process was to prepare single stranded DNA sequence to be altered. This was accomplished by harvesting single stranded plasmid from M13mp18 phage containing ET_AR, as described (120). The oligonucleotide containing the flag sequence was kinased and then annealed to the single strand DNA template. A second strand was synthesized using klenow fragment and T4 DNA ligase, in the presence of a mix of nucleotides and nucleotide analogs. The parent strand, not containing nucleotide analogues, was nicked and digested. A new second strand was polymerized completing a double-stranded plasmid, including the alterations contained in the mutant oligonucleotide.

Replacement of five serines in the third intracellular loop and carboxyl tail were accomplished using the same mutagenesis technique as was used in insertion of the flag epitope. A ET_AR clone containing the flag epitope was used as the template for mutagenesis. Three oligonucleotide primers were used (see figure 8) to mutate one or two serines at a time. Individual constructs were made that contained the flag sequence and the mutations contained in a single oligonucleotide primer. A construct containing all five mutated serines was made from one of the single site mutants. Each of the different constructs were sequenced using the dideoxy chain termination technique to

verify the presence of the anticipated mutations. It was also determined at a later time that the transfection of CHO-K1 cells with the mutant receptor did in fact contain this construct containing 5 serine to alanine alterations. This determination was made my PCR of the receptor from cell extracts, followed by restriction digestion with HaeIII and NarI, both of which cut at mutated sites, and not at wild-type, producing an additional fragment on digestion with either enzyme (121).

CELL CULTURE

All tissue culture dishes were manufactured by NUNC. CHO-K1 cells were a generous gift from L. Root and G. Shipley, and the rat A10 cells were a generous gift from B.E. Magun. A10 cells were grown in DMEM supplemented with 5% bovine calf serum and 10μg/ml gentamycin. The non-transfected CHO-K1 cells were grown in MCDB-302 media (Sigma) supplemented with 5% bovine calf serum, 10μg/ml gentamycin. Cells were stably transfected with the various constructs using the calcium phosphate technique (121). Following transfection cell clones were selected and grown in the media described with the addition of 700μg/ml G418 (Genetacin from Gibco).

^{125}I ET-1 BINDING EXPERIMENTS

Binding experiments were performed as described (110) on A10 vascular smooth muscle cells and stably transfected CHO-K1 clones of ETARjr, ETARjr-flag and ETARjr-flag/Δ5x (for details of receptors see System Construction in Results chapter). Confluent cells (about $5x10^5$) were incubated with 2.5nM ^{125}I ET-1 at 4°C for three hours in Hanks' balanced salt solutions containing 0.1% bovine serum albumen (HBSS+BSA). Following this incubation cells were extensively washed, solubilized in 1.0 N NaOH, and the cell-bound radioactivity was determined. Nonspecific binding was determined by binding in the presence of excess ($4x10^{-7}$M) unlabeled ET-1.

SATURATION OF ^{125}I ET-1 BINDING SITES

ETARjr-flag and ETARjr-flag/Δ5x expressing cells were incubated with 10 nM ET-1 in HBSS+BSA at 37°C for indicated times. Cells were then transferred on to ice and washed twice with ice cold HBSS+BSA, and incubated with 10 nM ^{125}I ET-1 at 4°C for three hours to saturate remaining receptors. Cells were extensively washed and solubilized in 1.0 N NaOH to determine cell-bound radioactivity.

^{125}I ET-1 BINDING AND INTERNALIZATION

Surface-bound and internalized ^{125}I ET-1 was determined using the acetic acid procedures as described by Resink et al. (108). Cells expressing ETARjr-flag and ETARjr-flag/Δ5x were incubated in HBSS+BSA with ^{125}I ET-1 at 4°C for three hours to saturate cell surface receptors. Cells were washed extensively to remove unbound ligand and then transferred to 37°C for specified times. At the end of the 37°C incubation ET-1 bound to the cell surface was removed by washing cells twice for 10 minutes each time at 4°C in 0.2M acetic acid (pH2.5) containing 0.5 M NaCl. Combination of the two washes constituted the acid-sensitive fraction of ^{125}I ET-1. The remaining cell-associated ^{125}I ET-1 was determined by solubilizing the cell layers in 1M NaOH, and constituted the acid-resistant fraction. Control experiments were done in which total bound ^{125}I ET-1 was determined by lysis without acid wash and compared to the summation of acid-sensitive and acid resistant counts.

FIXING AND STAINING CELLS FOR IMMUNOFLUORESCENCE.

Technique described in this section represents a combination of methods reported by (122, 123), and modified by C. Enns (personal communication). All receptor constructs expressed in CHO-K1 cells, grown in MCDB-302 media supplemented with 5% bovine calf serum 10μg/ml gentamicin, and 700μg/ml G418. Cells were grown on Fisher brand cover slips placed into NUNC 6-well plates. Cells were stimulated with

$1x10^{-8}$M ET-1 for stated periods of time at 37°C, and then washed twice with room temperature phosphate buffered saline (PBS). All remaining steps were carried out at room temperature. Paraformaldehyde was freshly diluted to 3% with PBS and incubated on washed cells for 15 minutes. Cells were rinsed two times with [PBS+10% goat serum] and then preincubated with [PBS+10% goat serum] for 15 minutes. Incubation with first antibody (Mouse 'M2' anti-flag IgG1 antibody diluted 1:50 in [PBS+10% goat serum]) was for 90 minutes. Cells were washed twice by dipping the cover slip into [PBS+10% goat serum]. Incubation with secondary antibody (goat anti-mouse IgG1 FITC diluted 1:150 in [PBS+10% goat serum]) was for 60 minutes. Cells were then washed twice by dipping the coverslip into [PBS+10% goat serum], and then one final wash in PBS. The final product was then mounted on slide with 0.2% p-phenylenediamine/50% glycerol.

INOSITOL PHOSPHATE ASSAY

This technique was performed according to the protocol described by (124). Cells were grown to near confluence (~$3x10^6$ cells/plate) on 6cm NUNC plates, and serum deprived (0.1% bovine calf serum) in appropriate media containing 1μCi/ml [^{3}H]*myo*-inositol (Dupont/NEN) for 24 hours. After removal of the labeling medium, the cells were rinsed, and then incubated in serum free media without myo-inositol. Cells were then treated as described for specific experiments. Over the period that inositol phosphates were to be measured LiCl was added to the media at a final concentration of 100mM to inhibit the degradation of the second messenger by a phosphatase. Reactions were terminated by placing the cells on ice and adding 1ml 0.3M formic acid for 20 minutes, and then 0.35ml NH_4OH (0.4M), and brought up to 5ml with dH_2O. Samples were loaded on formic acid-conjugated form of Dowex-1 ion exchange resin (Sigma 1x8-

400). Samples were then washed and IP1, IP2, and IP3 were eluted in a single fraction with 0.1M formic acid/ 0.75M ammonium formate, and quantified by liquid scintillation counting.

XENOPUS OOCYTE EXPRESSION OF RECEPTORS

ET_ARjr-flag and ET_ARjr-flag/Δ5x mRNA were transcribed from 2μg of linearized plasmid and capped with 7mGpppG using T7 RNA polymerase (from BRL), and resuspended in 25μl RNase free dH_2O. Isolation of ovaries, and isolation and injection of oocytes were carried out by personnel in J.P. Adelman's laboratory at the Vollum Institute for Advanced Biomedical Research. Three to seven days after injection of *in vitro* transcribed mRNA, cells were voltage-clamped at -60mV and currents were recorded in response to various doses and periods of ET-1. Experiments were done at room temperature using a standard two-microelectrode voltage-clamp in ND96 (96mM NaCl, 2 mM KCl, 1mM $MgCl_2$, 5mM Hepes, and 1.8mM $CaCl_2$, unless calcium free, in which case no $CaCl_2$ was added).

Desensitization experiments were done by stimulating the oocytes with 10nM ET-1 in ND96 for one minutes and recording the response. The oocytes were then washed constantly for 30 minutes. They were then restimulated with the same dose and duration as the first. Current recordings were recorded throughout.

IMMUNOPRECIPITATION

The following is the initial protocol used for immunoprecipitation which was altered stepwise as results dictated (125). Preliminary immunoprecipitations were carried out on cells metabolically labeled with ^{35}S methionine, in an attempt to optimize the recovery process. There are 15 methionines in each receptor protein which was expected to provide adequate labeling for following. Cells were grown as described in Materials and Methods. Cells were washed with room temperature PBS, and then placed in

DMEM deficient in methionine. The cells were incubated in this media for one hour prior to labeling for two additional hours in methionine free DMEM supplemented with 100mCi/ml ^{35}S-methionine, and 0.1% V/V bovine calf serum. At the end of the labeling period cells were washed twice with ice cold PBS, and then solubilized in Lysis buffer #1 (see below). Lysates were then clarified by centrifugation at 16,000 x g (12,000 rpm in epindorff centrifuge) for five minutes. The supernatant was then transferred to a fresh tube and preabsorbed with 50µg/ml of Protein G sepharose or protein A sepharose, rotating at 4°C for 1 hour. The sample was then spun at 12K RPM for two minutes to pellet the sepharose. To the supernatant was added 24µg of mouse anti flag IgG1 M2 antibody, and rotated at 4°C overnight. The primary antibody was then precipitated out by adding 40ml protein G sepharose or protein A sepharose and rotating at 4°C for an additional hour. The sample was then pelleted (same as above), then suspended in 1ml of lysis buffer #1, and repelleted. This wash procedure was done three times. The pellet was then suspended in 100µl lysis buffer #1 and layered on top of 1ml of washing buffer with 15% sucrose (see below). The sepharose/M2/antigen complex was then pelleted through the wash solution for two minutes at 12K RPM. This final pellet was suspended in 100µl 2X Laemmli buffer (see below), heated to 95°C for 5 minutes, and then loaded on a 10% polyacrylamide SDS gel (gel made according to (120)). The samples were run out overnight, and then the gel was soaked in Coomassie stain for 30 minutes, followed by destain as needed, and finally soaked in Amplify for 15-30 minutes. Gel was dried with heat and then exposed to film.

Lysis buffer #1 contained 50mM Tris-Cl pH 8.0, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 40µg/ml bestatin, 50µg/ml leupeptin, and 1mM PMSF. Washing buffer with sucrose contained 50mM Tris, 150mM NaCl, 1% SDS, 1% sodium deoxycholate, all pH to 8.5 with HCl, and then 1.5 g sucrose per 10 ml buffer. 2X laemmli buffer contained 0.125M tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% beta-mercaptoethanol, and 0.002% bromphenol blue.

The experiment described above gave poor labeling of cellular proteins. Therefore, additional experiments were carried out, testing different incubation periods. Control samples include the comparison of ET_ARjr cell lysates, with those of ET_ARjr-flag. Another control involved the competition of specific binding with 10μg/ml soluble flag peptide (from IBI/Kodak). This time cells were either methionine deprived for one hour, followed by a four hour labeling, or just labeled overnight without prior deprivation. The resulting autoradiogram of this experiment showed no bands that were unique to cells expressing the flagged receptor. One consideration is that the ET_AR is proteolytically cleaved in a similar fashion to the ET_BR, which is prevented in the presence of EDTA (126, 127). Precipitation carried out on cells lysed in lysis buffer #1 that included 50mM EDTA gave results identical to those done without the chelator. Another possibility raised was that the long primary precipitation could be contributing to the loss of receptor protein. To test this question precipitations were carried out for either one hour or overnight. The result of this experiment was that even the short incubation was long enough to bind the cross reacting-proteins, but still did not result in the presence of a receptor being precipitated.

At this time it seemed possible that the M2 antibody was not binding the antigen with high enough affinity, and therefore was being washed off prior to the final stages. Personal communications with S. Malloy (in G. Thomas' lab) revealed that the M1 antibody purchased from IBI/Kodak was much more efficient at precipitating proteins containing the flag epitope. I did a comparison of the M1 (15μg/ml) and M2 (24μg/ml) antibodies at precipitating labeled cellular proteins. For this experiment no EDTA was used, and because of M1's calcium requirement 1mM $CaCl_2$ was included in all solutions used in the processing of M1 samples. Comparing the precipitates of these two antibodies showed that M1 had a higher background, but neither gave a specific band that could be associated with the receptor.

The lack of precipitation of the ET_ARjr-flag led us to believe that the epitope was not being recognized by the antibody under these conditions. The precipitation was then done on samples that had been lysed in lysis buffer #1 that had 3% SDS, rather than the normal 1%. These samples were heated to 65°C for 15 minutes to assure denaturation of any secondary structures that were interfering with antibody antigen interactions. These were cooled to room temperature and diluted so that the final SDS was 1% before the primary antibody was added. When this technique failed to give the specific band other lysis conditions were tried. Two different detergents, each at two different PHs were tested. The detergents were 0.5% triton X-100 and 1.0% n-Octyl β-D glucopyranoside. Each of these were prepared in 10mM Tris, 150mM NaCl, 5mM EDTA, 40μg/ml bestatin, 50μg/ml leupeptin and 1mM PMSF, with a portion of each adjusted to a pH of 7.0 or 8.0. These conditions also failed to precipitate the receptor.

The laboratory of M. Chinkers was having success precipitating a flagged protein with the M2 antibody. I therefore used conditions identical to those used in their lab (125), including the use of their reagents (generously given to me by the Chinkers' lab). In this experiment I also compared the precipitation using 1μg or 24 μg of M2 antibody. This technique proved no more useful than the others tested previously.

A probable explanation for the lack of results was that the commercial preparation of antibody was unable to precipitate the receptor under the conditions tested. A test of this hypothesis was to attempt do the precipitation using serum containing polyclonal antibody made against the peptide sequence of the flag epitope in rabbit. This experiment involved the comparison of untransfected CHO-K1 cells to those cell clones expressing ET_ARjr, ET_ARjr-flag, and ET_ARjr-flag/Δ5x. Each of these were done in the presence and absence of soluble flag competitor. Although the bands that cross-react with this serum were different from those that cross-reacted with the M2, no bands were seen in the samples from cells expressing flag compared against those samples from cells lacking the epitope.

CHAPTER 3
RESULTS & DISCUSSION

CONSTRUCTION OF MODEL RECEPTOR SYSTEM

Since the discovery of the endothelin family of peptide hormones, a large number of studies have tried to determine ET's role in physiology. One of the most remarkable features of ET_AR is its profound desensitization in response to ET-1. Perturbation of desensitization would likely lead to a disease state. Hence, learning more about the regulation of this system under normal conditions would be informative, and might lead to discoveries about the role of ET in pathophysiology.

The role of phosphorylation in the regulation of desensitization of the endothelin receptors has been investigated. *In vitro* tests show that chemical activation of protein kinase C (PKC) artificially induces desensitization (101, 103, 118). The evidence has been inconsistent however, sometimes showing PKC inhibitors block desensitization, and other times no effect is seen (102, 106, 107). These data suggest a role for PKC, but leave undetermined the target of the kinase which is responsible for the cellular effects. In my opinion, the most direct way to test the role of phosphorylation of the ET_AR would be to compare normal receptors to those lacking the amino acid targets of the kinases. This would eliminate phosphorylation of the receptor without chemically affecting other cellular functions.

RECEPTOR CLONING

Isolation of Human ET_AR cDNA

The gene encoding the ET_AR was first cloned from rat sources (37). Hosoda later cloned and expressed the human ET_AR cDNA (38). Reports of cloning the ET_AR included Northern blot analysis to determine tissue distribution. Heart and lung tissue consistently demonstrated the highest levels of expression. Human cadaveric lung was

used as a source of ET_AR mRNA because lung tissue was consistently demonstrated to express high levels. Total RNA was isolated using the guanidine isothiocyanate acid phenol method (119). This RNA was reverse transcribed to make cDNA which was then used as the template for polymerase chain reaction (PCR) amplification of the ET_AR (128). Primers for this reaction were chosen based on the published sequence of the human gene (see figure4) (38). The PCR product was subcloned using the HinDIII and XhoI restriction endonuclease sites. The resulting fragment was subcloned into Bluescript (pBSIIKS+ , Stratagene, La Jolla, CA). Sequencing of the clone was accomplished using the dideoxy chain termination method (129). The DNA sequence obtained using this method was found to be identical to the published sequence (see figure 5) (40). The cDNA clone of the receptor was designated ET_ARjr. In order to test function, the ET_ARjr coding sequence was subcloned into the pcDNA I/neo expression vector (Promega). Transcription of the receptor in this construct is driven by a cytomegalovirus (CMV) promoter (see figure 6). This construct was chosen because it simplified transfection into mammalian cells by co-expressing the neomycin resistance gene, which was useful as a selectable marker.

CLONE MANIPULATION

Insertion of Flag Epitope

Antibodies provide a powerful tool for the molecular study of proteins. Antibodies to the endothelin receptor would permit evaluation of subcellular localization. Immunoprecipitation of the receptor for evaluation of molecular weight and phosphorylation states would also be possible with antibodies. In order to study the receptor using these techniques antibodies would need to be made against the receptor, or the receptor would have to be altered so it would be recognized by already available antibodies. It was decided that insertion of an epitope recognized by an available, previously characterized antibody would be the most productive method. Commercially

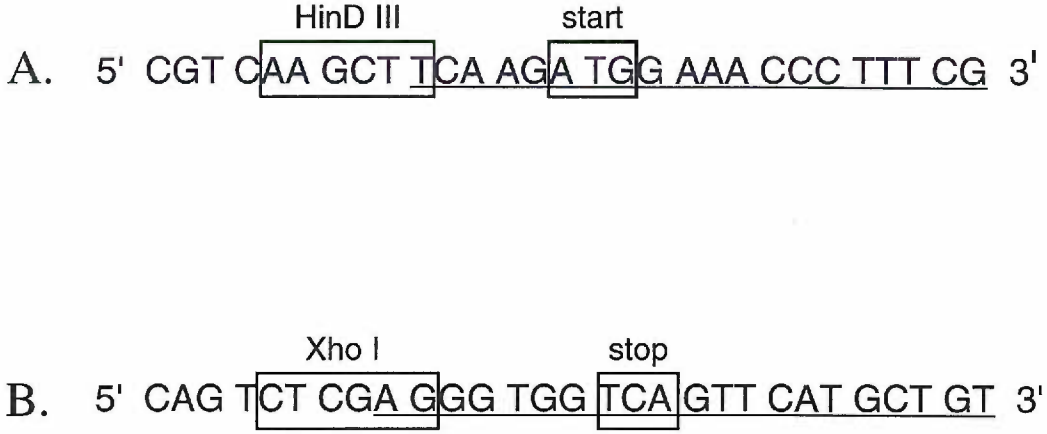


Figure 4. Primers used to amplify the human endothelin-A receptor using PCR techniques. "A." represents the 5' primer which includes the translational start site, and an engineered HinD III restriction endonuclease site. "B." represents the 3' primer which includes the translational stop site, and an engineered Xho I restriction endonuclease site. Underlined bases represent homology with the human endothelin-A receptor cDNA sequence. Primers were given the designations #156 & #157 respectively.

Figure 5. Nucleotide and amino acid sequence of the human ETRA. Putative transmembrane domains (1-7) are enclosed in black boxes. The "flag" sequence inserted as shown boxed in blue box. ● are potential N-glycosylation sites. Red boxes are serines that have been mutated in specific constructs. Amino acids are numbered at line ends, and below mutated serines.

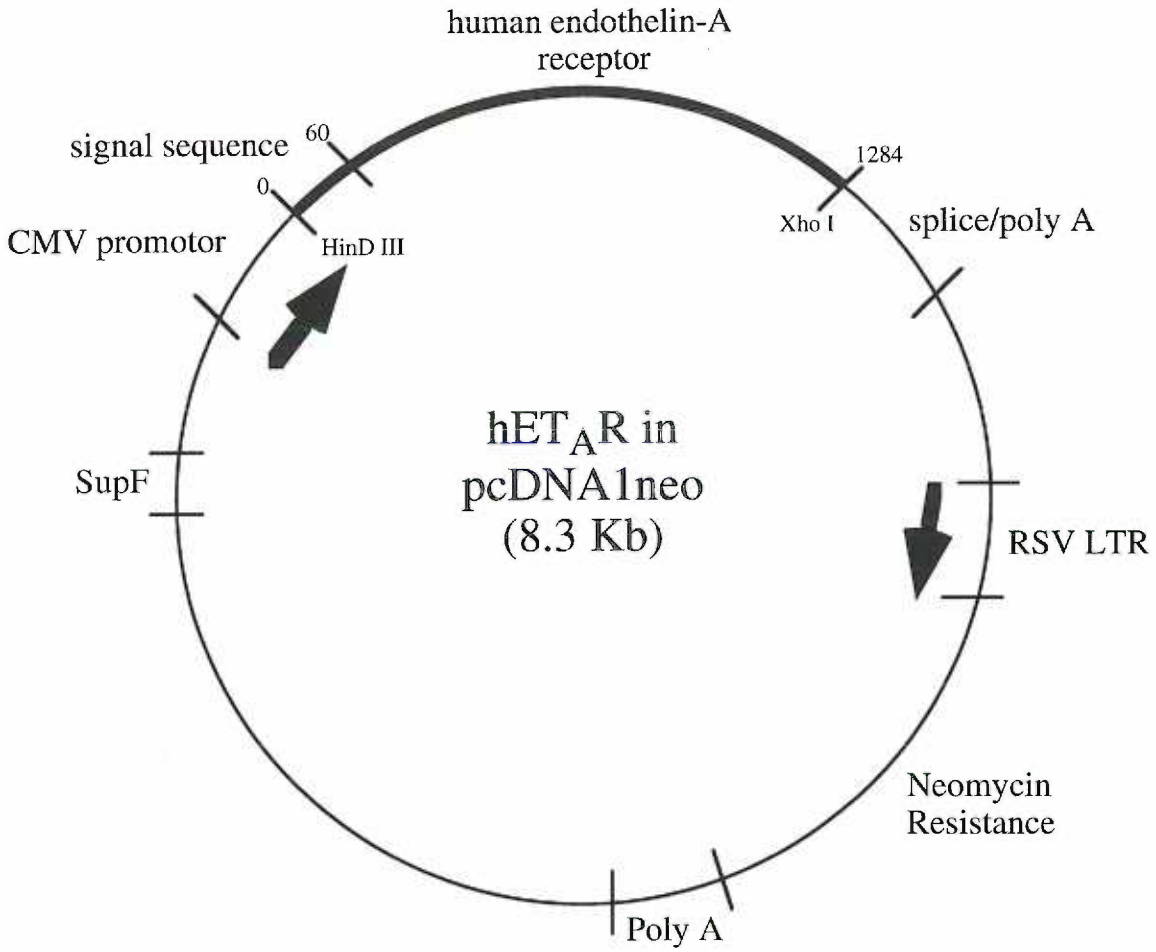


Figure 6. Organization of the human endothelin-A receptor subcloned into the pcDNA1neo expression vector (from Invitrogen). Numbers represrent nucleotide distance from translational start site. Thickened line represents the ET_AR sequence cloned into the Hind III and Xho I sites of pcDNA1neo expression vector.

available monoclonal antibodies (IBI/Kodak) recognize a defined epitope of the amino acid sequence [Asp-Tyr-Lys-Asp] (130). This sequence is part of the "flag" epitope. The greatest advantage of this sequence is that it is short (8 amino acids), and therefore unlikely to interfere with important structural aspects of the receptor (see figure 7). Another advantage to this epitope is that it contains the amino acid sequence cleaved by enterokinase, which is potentially useful for protein purification or elimination of cell surface epitopes. Twenty-four base pairs coding for the eight amino acids were inserted immediately 3' to the putative signal peptide (see figures 5 and 7). Prediction of the sleavage site for the signal sequence of the ET_AR was used to position the flag to become the new extracellular N-terminus of the receptor (40). Insertion into the cDNA was accomplished using an oligonucleotide directed mutagenesis system (Amersham), which primes a single stranded plasmid with an oligonucleotide containing the desired mutation. The parent strand is then digested, leaving only the newly polymerized DNA. A second strand is then primed from the remaining single stranded DNA, resulting in a complete plasmid with site-specific mutations.

The ET_ARjr construct was subcloned into the m13mp18 plasmid because mutagenesis requireed a single-strand plasmid template. The new construct was partially sequenced to verify the fidelity of the insertion. Once mutated, the cDNA sequence was then recloned back into pcDNAI/neo, and was given the designation ET_ARjr-flag.

Site Directed Mutagenesis

Several G-protein coupled receptors are known to be phosphorylated in response to hormone binding. Phosphorylation has been associated with desensitization (62, 64, 66, 131). The most direct way to determine if phosphorylation is involved in desensitization is to alter the potentially phosphorylated sites in the receptor. Comparison of the mutant receptor with a non-mutant form will reveal the role of phosphorylation in the desensitization process. The oligonucleotide directed mutagenesis kit (from

A. 5' TGT GTA ATC AGT GAT TAC AAG GAC GAC GAT GAC AAG ATC AAT CCT GAG AGA TAC 3'

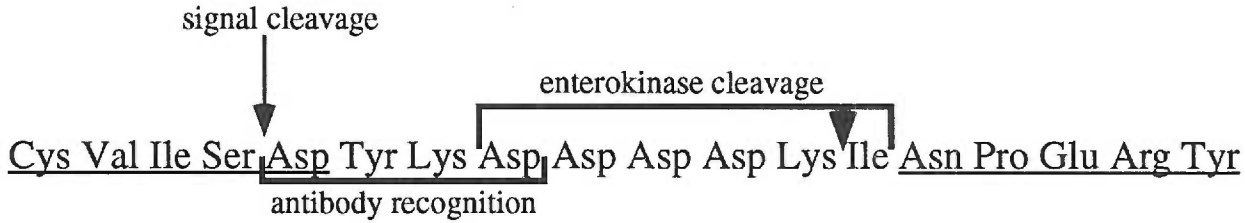


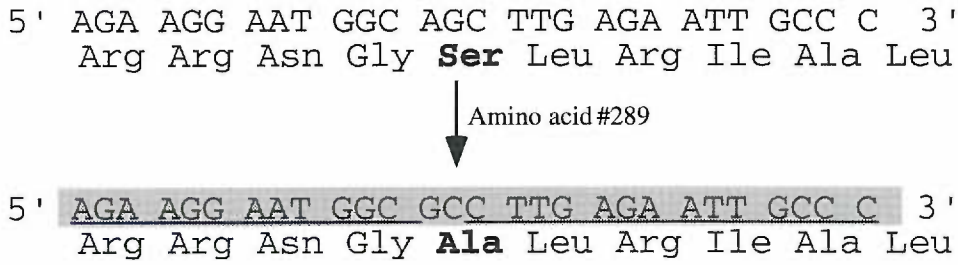
Figure 7. Insertion of the flag epitope. "A." represents the sequence of the oligonucleotide primer used in site directed mutagenesis to insert the "flag" epitope. The underlined bases represent homology with human endothelin-A receptor. "B." represents the translated sequence that resulted from the insertion, including antibody recognition and enterokinase cleavage sequences . The underlined amino acids represent homology with the human endothelin-A receptor. The oligonucleotide primer was given the designation #173.

Amersham) was used to construct the mutants. The ET_ARjr-flag was used as the template so all of the constructs would contain the flag epitope. The mutations were directed at the consensus protein kinase C/CaM kinase II phosphorylation sites (see table 3). One target for mutagenesis is a single serine located in the third intracellular loop (Ser-289 between transmembrane domains 5 & 6). The other two target sites each contain two serines and are located near the C-terminus of the receptor (Ser-391 & Ser-393 or Ser-420 & Ser-421 distal to the 7th transmembrane domain). All selected serines were mutated to alanines. ET_ARjr-flag constructs were made which contain each of the mutated targets, as well as an additional construct which contains all three target mutated sites (5 serines in total). The clones were named based on the number given to the oligonucleotide used to mutate the ET_ARjr-flag sequence. Therefore the S289A mutant was called ET_ARjr-flag/Δ176, the S391A+S393A mutant was called ET_ARjr-flag/Δ219, and the S420A+S421A mutant was called ET_ARjr-flag/Δ225. The S289A, S391A, S393A, S420, S421A combined mutant construct was called ET_ARjr-flag/Δ5x (see figures 5 and 8).

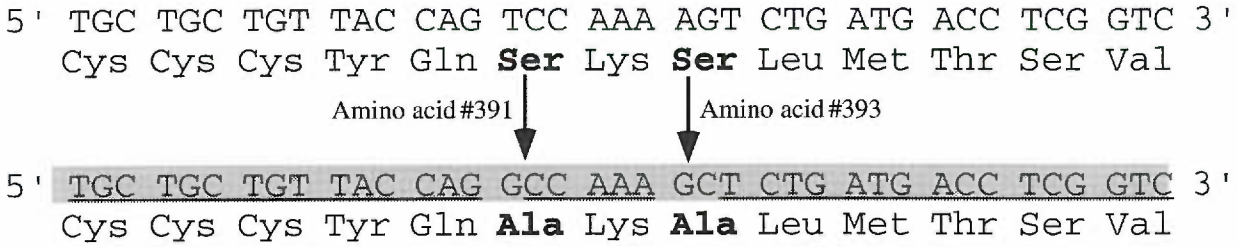
Protein Kinase C	MOTIF	REPORTS
	S/T-X-K/R	20
	K/R-X-X-S/T	13
	K/R-X-X-S/T-X-K/R	7
	K/R-X-S/T	10
	K/R-X-S/T-X-K/R	6
calcium calmodulin kinase II	R-X-X-S/T	

TABLE 3. Phosphorylation sites of PKC. S/T represent serine or threonine (which are phosphorylated), X represents any amino acid, K/R represents lysine or arginine. Reports represents number of times the specific sequence has been reported to be phosphorylated (132). The calcium calmodulin kinase II consensus sequence was reported by Kennelly and Krebs (117).

A. Oligo #176



B. Oligo #219



C. Oligo #225

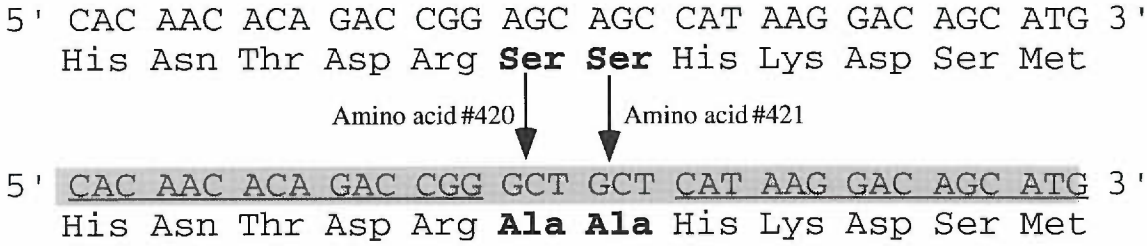


Figure 8. Oligonucleotide primers used in the mutation of cytoplasmic serines. The top sequence for each oligo represents the base composition and protein sequence of the ET_AR cDNA. Amino acids affected by the mutations are in bold type. The sequence of the oligonucleotide primer used in site directed mutagenesis is shaded, and the underlined bases represent homology with ET_AR.

STABLE TRANSFECTIONS

CHO-K1 cells (ATCC CRL 9618) were transfected with each of the six different constructs of the ET_AR (cloned into pcDNAI/neo) using the calcium phosphate technique (121). These were designated CHO-K1[ET_ARjr], CHO-K1[ET_ARjr-flag], CHO-K1[ET_ARjr-flag/Δ176], CHO-K1[ET_ARjr-flag/Δ219], CHO-K1[ET_ARjr-flag/Δ225], and CHO-K1[ET_ARjr-flag/Δ5x]. Individual clones were selected for resistance to G418 (also known as geneticin or neomycin). The CHO-K1 cell line was chosen as the expression system because of its low background reactivity to ET-1, and because rat ET_AR is functional when expressed in those cells (42). Two cell clones of each construct were chosen based on their inositol phosphate (IP) accumulation in response to ET-1 exposure. This was determined using [3H]myo-inositol labeled cells, as described (124). All selected clones accumulated IPs at levels five to 15 times that of non-expressing controls.

CHARACTERIZATION OF RECEPTOR MODEL

The receptor constructs were stably transfected into CHO-K1 cells. Cell clones were isolated based on resistance to neomycin, and accumulation of IPs in response to ET-1 exposure. Although normal levels of IP were recorded, cell clones may have a greater number of receptors with lower activity, or fewer receptors with higher activity levels than the control. It is also possible that molecular manipulations altered the receptor's affinity for ligand. Therefore it was important to verify that the affinity of the cloned receptor constructs did not differ from receptors expressed naturally. Another concern was that modifications had changed the receptor's ability to signal. The model tested here predicts that regulation of mutants would be different from wild-type, but binding and transduction mechanisms would not.

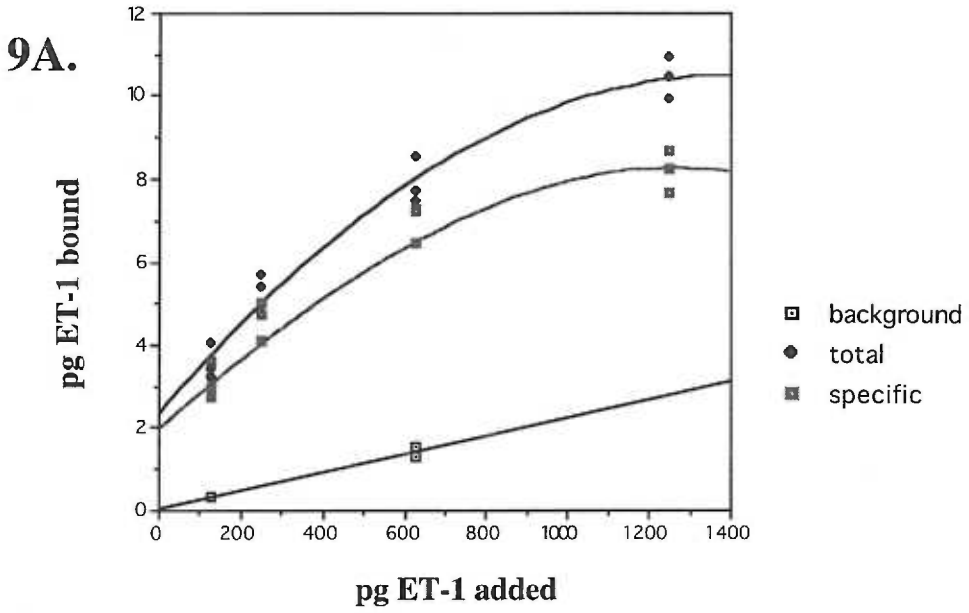
Receptor constructs were characterized in CHO-K1 cells and in *Xenopus* oocytes. The CHO-K1 cells are useful because they are mammalian in origin, and can be manipulated easily over the course of hours. Electrophysiology studies in Xenopus

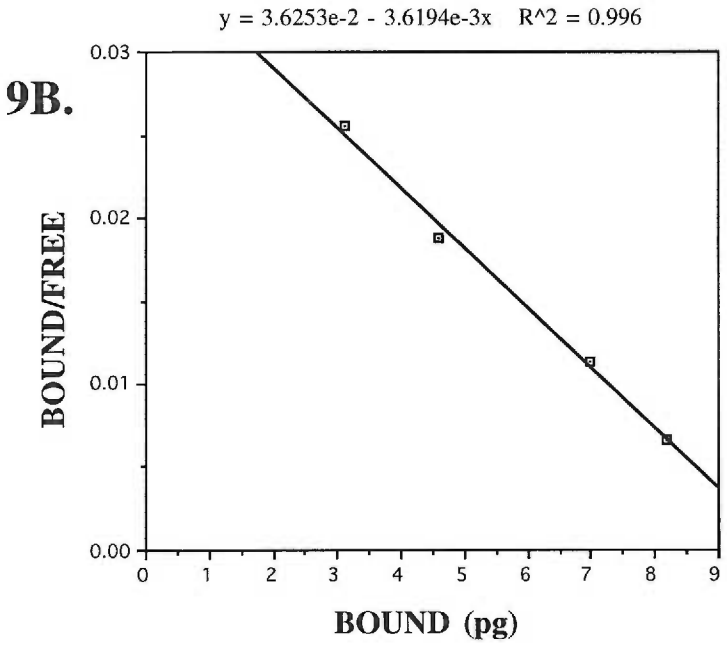
oocytes measure the movement of charged ions across the plasma membrane and are able to discern discrete events that occur over the period of seconds.

ET_AR AFFINITY FOR ET-1 IN CHO-K1 CELLS

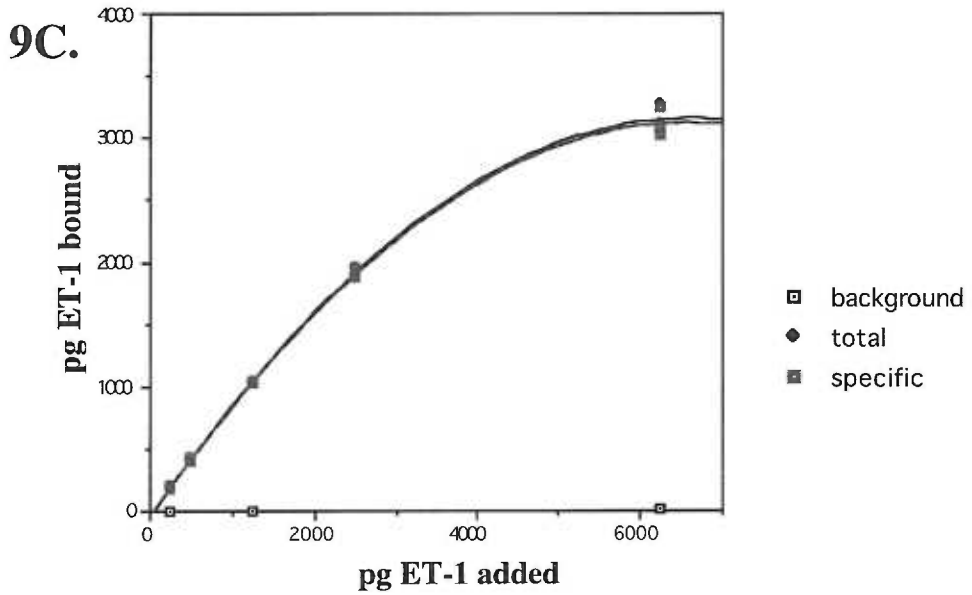
To address the question of cloned receptor affinity, binding studies of radiolabeled ET-1 were carried out. The first analysis was done to characterize an endogenous ET_AR expressed on a rat vascular smooth muscle cell line (A10 ATCC CRL 1476). ^{125}I ET-1 bindings were done as described in Materials and Methods, and are a variation of the method described (110). Binding analysis was accomplished by incubating cells with varying concentrations of ^{125}I ET-1, and allowing the binding to reach equilibrium by binding at 4°C for 3 hours. Binding done in the presence of excess unlabeled ET-1 represented the non-specific binding of label to the cells. Scatchard analysis of the data derived from the A10 cells revealed that they express a single high affinity receptor for ET-1 with a Kd of about 100pM, and a Bmax of 14,000 receptors/cell (see figure 9a and 9b). This receptor density and affinity are consistent with other reports of cultured rat vascular smooth muscle cells (110, 133).

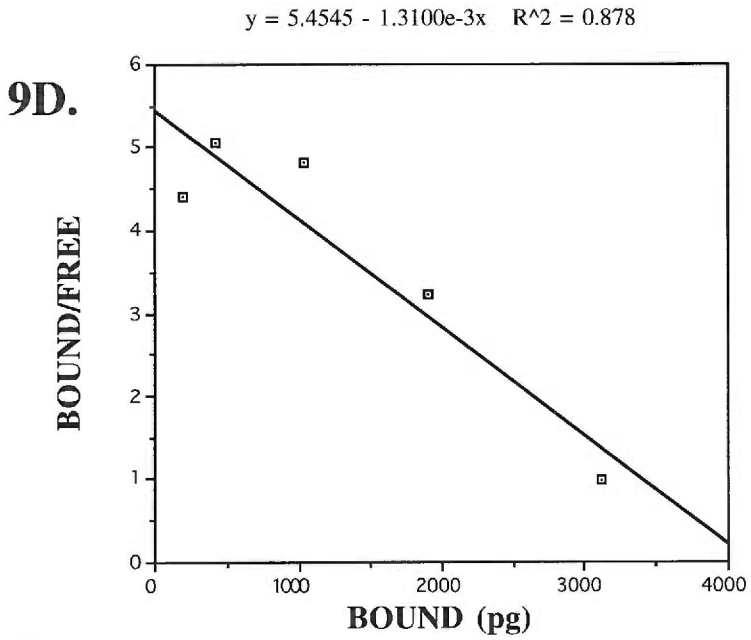
It was necessary to show that the affinity of the various constructs for ET-1 had not been affected by the manipulations of the receptor sequence. The insertion of eight amino acids in the amino terminus of ET_ARjr-flag, and subsequent mutations of the five serines were both tested for changes in affinities. Binding was carried out identically to that described for A10 cells. Scatchard analysis of the CHO-K1[ET_ARjr-flag] cells revealed a Kd of 220pM, and a Bmax of $1.3x10^6$ receptors per cell (see figure 9c and 9d). The Scatchard analysis of ET-1 binding to CHO-K1[ET_ARjr-flag/Δ5x] cells revealed a Kd of 300pM, and a Bmax of $2.8x10^6$ receptors per cell (see figure 9e and 9f). Although these cells express a higher density of receptors on the cell surface, their affinity for ET-1 is unaffected by the flag epitope.



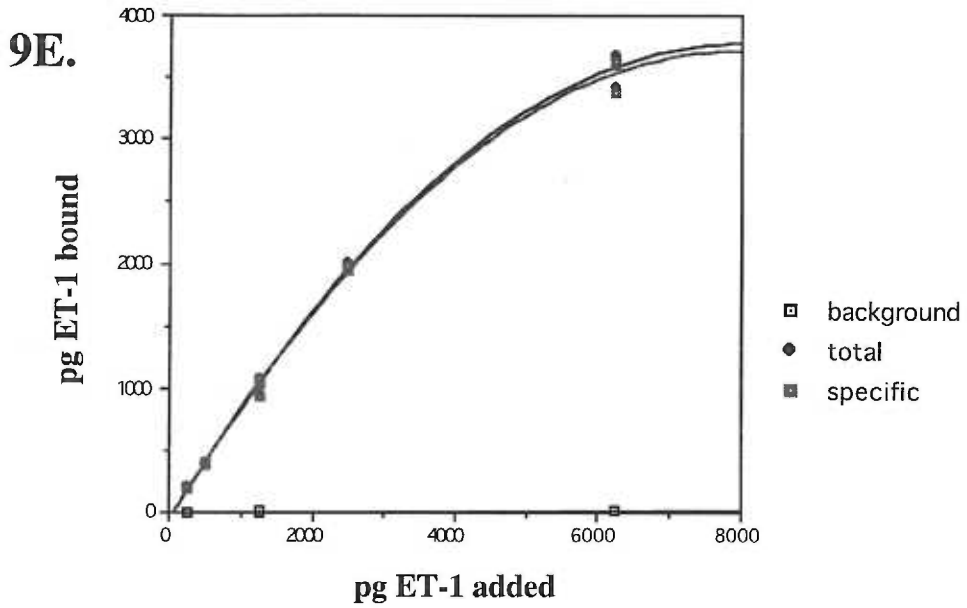


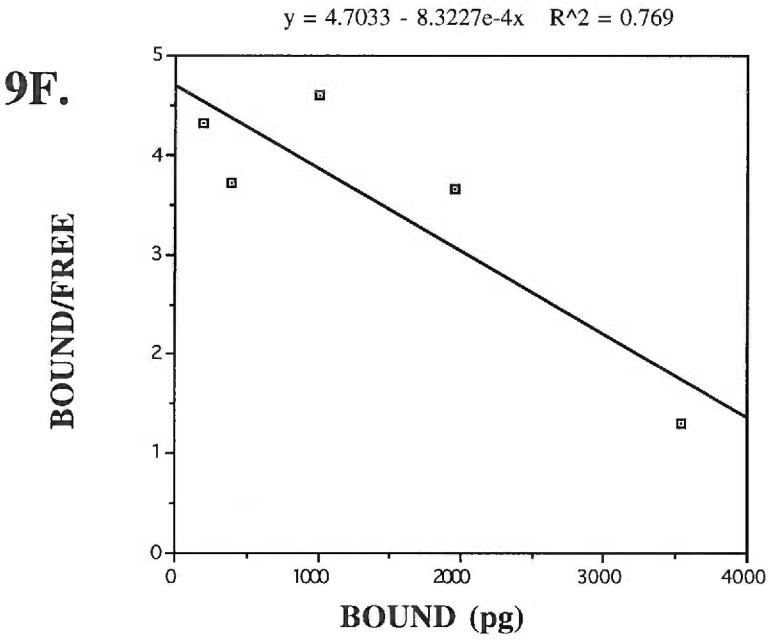
Figures 9A and 9B. Binding and Scatchard analysis of A10 cells. A. Total binding of ET-1 to Vascular smooth muscle cells (A10) was determined by incubating $1.5x10^5$ cells per well with specified concentrations of ^{125}I ET-1 for 3 hr. at 4°C, followed by a wash and lysis. Background binding was determined in the presence of $4x10^{-7}M$ unlabeled ET-1. Subtraction of background from total binding provided specific binding. B. Scatchard plot of data obtained in A. providing the "best fit" line, the equation of which is provided.





Figures 9C and 9D. Binding and Scatchard analysis of ETRAjr-flag. C. Total binding of ET-1 to CHO-K1 cells expressing ETRAjr-flag was determined by incubating $7.5x10^5$ cells per well with specified concentrations of ^{125}I ET-1 for 3 hr. at 4°C, followed by a wash and lysis. Background binding was determined in the presence of $4x10^{-7}M$ unlabeled ET-1. Subtraction of background from total binding provided specific binding. B. Scatchard plot of data obtained in A. providing the "best fit" line, the equation of which is provided.





Figures 9E and 9F. Binding and Scatchard analysis of ETRAjr-flag/Δ5x. A. Total binding of ET-1 to CHO-K1 cells expressing ETRAjr-flag/Δ5x was determined by incubating $4.0x10^5$ cells per well with specified concentrations of ^{125}I ET-1 for 3 hr. at 4°C, followed by a wash and lysis. Background binding was determined in the presence of $4x10^{-7}M$ unlabeled ET-1. Subtraction of background from total binding provided specific binding. B. Scatchard plot of data obtained in A. providing the "best fit" line, the equation of which is provided.

The ET-1 affinity calculated for the ET_ARjr constructs was consistent not only with endothelin receptors expressed in vascular smooth muscle cells, but with those expressed natively in a variety of cell-types, as well as cloned receptors derived from rat, bovine, and human sources (37, 41, 42). Most of the Kds reported for ET_AR vary over a range from 100pM to 400pM (12). Normal Kds provided evidence that ET_ARjr-flag and ET_ARjr-flag/Δ5x represent normal endothelin-A receptors, and that neither the cloning nor the serine manipulations have significantly affected the receptor's affinity for ligand.

RECEPTOR SIGNALING

The data reported above supports the idea that mutations did not affect the receptor-ligand interactions. Subsequently, it was demonstrated that transmembrane signaling was also intact. This was shown both in *Xenopus* oocytes and in CHO-K1 cells expressing the different receptor constructs (see Materials and Methods for details of techniques used for oocyte expression). Electrophysiology recordings from these cells provided an opportunity to monitor cellular activation at the ionic level, which correlates to receptor activity. The real-time current across the cell membrane is represented by these measurements (134).

DOSE RESPONSE

Tissue culture systems were also used to characterize signaling in the different constructs. Responses were measured over a range of ET-1 doses. IP accumulation was measured over the first 15 minutes of stimulation with doses ranging from 1pM to 1nM of ET-1 (see figure 10). Each dose-response data point represents a naive batch of cells with no previous stimulation with ET-1. Results not only revealed that the relative responses of ET_ARjr-flag and ET_ARjr-flag/Δ5x were identical, but also that near 100nM ET-1 elicits a 50% maximal response (EC_{50}). The range of responses to ET-1 and the

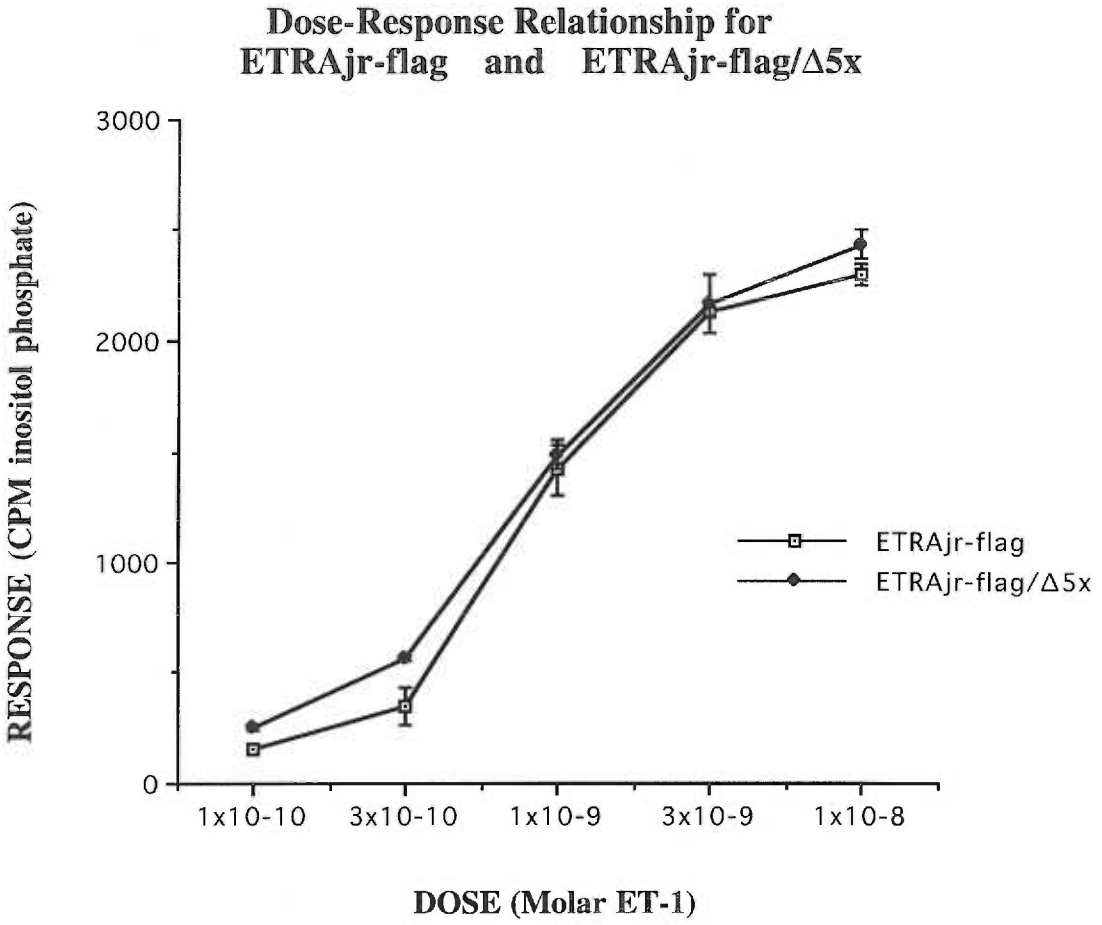


Figure 10. Dose-response comparison of ETRAjr-flag and ETRAjr-flagΔ5x constructs. CHO-K1 cells expressing either ETRAjr-flag or ETRAjr-flagΔ5x were prelabeled with [^{3}H] myoinositol (a substrate for IPs). Cells were stimulated with specified dose for fifteen minutes in the presence of 100mM LiCl and then lysed and assayed for IPs as described in Materials and Methods. Each point represents the mean of triplicate samples, plus or minus standard deviation.

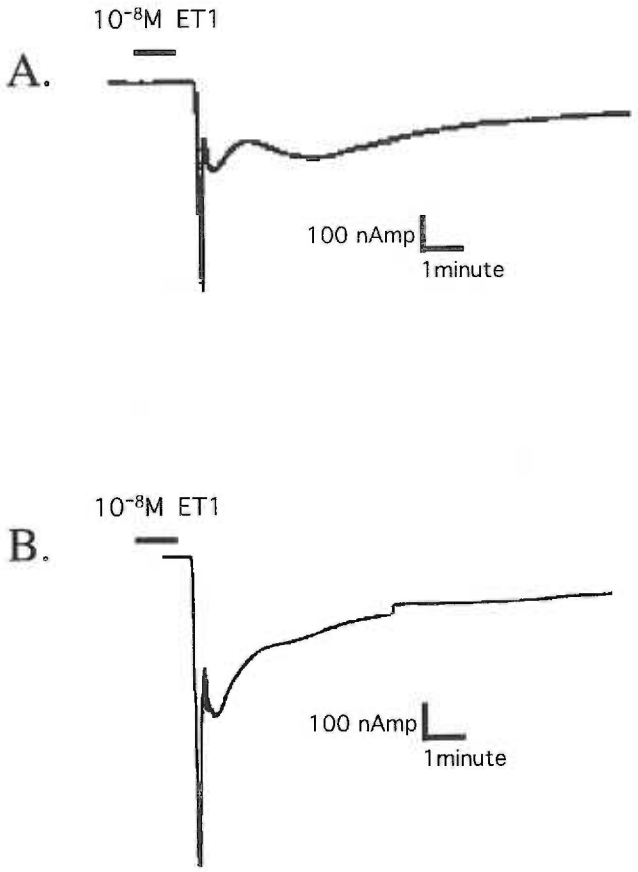
EC_{50} are consistent with reports for transfected CHO cells, as well as native receptors in mesangial, glial and endothelial cells (135-138).

ELECTROPHYSIOLOGY IN *XENOPUS* OOCYTES

Oocytes expressing ET_ARjr-flag and ET_ARjr-flag/Δ5x constructs were tested for response to ET-1 stimulation. Signaling was measured three to seven days after injection of mRNA. The current was recorded while cells were voltage clamped at -60mV. Different constructs tested gave equally strong responses, lasting for similar lengths of time (see figure 11a and 11b). The current tracings revealed a biphasic response. The first phase was a rapid onset spike of inward current of approximately 1μAmp, which lasted about one minute. This was followed by a slowly developing inward current of 200-300μAmps which lasted for 20-30 minutes. When cells were bathed in calcium free solution the first phase of the response was unaffected. The current flux, however, returned to baseline and showed no signs of a secondary rise (see figure 12). The sources of the two phases appeared to be internal stores and extracellular environment respectively. The flow of these currents reversed direction when the voltage clamp was -25 to -35mV. This reversal potential is characteristic of a calcium-dependent chloride channel (139). All measurable parts of the current tracings from the various systems used were indistinguishable between the constructs.

DISCUSSION

The binding data indicate that the ET_ARjr construct expressed in transfected CHO-K1 cells is structurally and functionally similar to ET_AR expressed naturally in vascular smooth muscle cells. Therefore, this construct can be used as the template from which various altered constructs can be derived. The first variation was the insertion of the flag epitope to produce ET_ARjr-flag. Altering the amino terminus of the receptor did



Figures 11A and 11B. Xenopus oocytes expressing A. ETRAjr-flag or B. ETRAjr-flag/Δ5x. Oocytyes injected with *in vitro* transcribed mRNA 3-7 days prior were voltage clamped at -60mV using two electrodes. These clamped cells were then exposed to $1x10^{-8}$M ET-1 for one minute. Inward current flux is represented as a downward deflection with an intensity represented by the scale markings given.

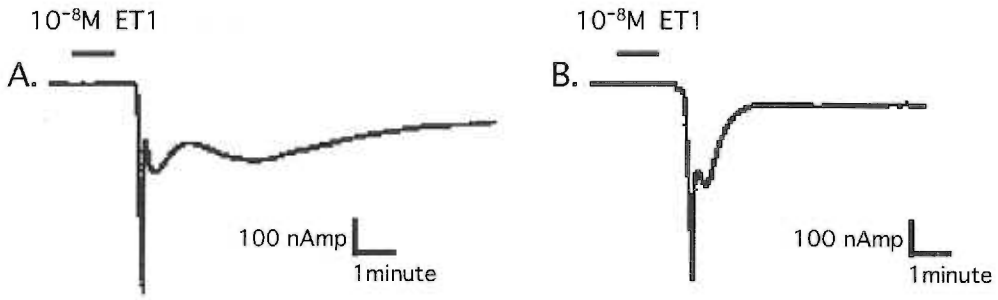


Figure 12a and 12b. Xenopus oocytes expressing ETRAjr-flag with and without extracellular calcium. Oocytyes injected with *in vitro* transcribed mRNA 3-7 days prior were voltage clamped at -60mV using two electrodes. These clamped cells were exposed to $1x10^{-8}$M ET-1 for one minute in A. normal ND96 containing calcium, or B. calcium-free ND96. Inward current flux is represented as a downward deflection with an intensity represented by the scale markings given.

not negatively affect ligand binding, evidenced by the near identity of measured dissociation constants with normal controls.

The mutation of multiple cytoplasmic serines was intended to test their role in receptor desensitization. Mutation of the selected amino acids did not affect the receptor's affinity for ligand, evidenced by the normal Kd. It was also revealed that signaling is unaffected by manipulations of protein sequence because the IP dose-response curves of ET_ARjr-flag and ET_ARjr-flag/Δ5x were identical. Signaling was identical between the two receptor constructs in magnitude and duration in the Xenopus oocyte expression system.

These data demonstrate that ET_ARjr-flag and non-flagged receptors are functionally indistinguishable from one another. The ET_ARjr-flag will be used as the "normal control" against which mutants will be tested. The binding and signaling data also demonstrate the integrity of the ET_ARjr-flag/Δ5x construct. This mutated receptor has wild-type affinity for ligand binding, signaling through IPs, and appropriately activates ion flux in *Xenopus* oocytes. With affinity and signaling unaffected, comparison of ET_ARjr-flag and ET_ARjr-flag/Δ5x should reveal the role of the five serines in desensitization.

An additional CHO-K1 cell clone was characterized for each receptor construct in order to ascertain which clones were best suited for this system. Binding studies revealed that the alternate clones expressed either equal or greater numbers of receptors than those previously tested (data not shown). Characterization therefore proceeded with existing clones, but with the caveat that the receptor levels are high. If the density of receptors found on these clones interfered significantly with some aspect of regulation, differences would have been discovered that were unaccounted for by their molecular alterations. This was not the case. There are $5x10^5$ receptors per cell on the CHO-K1[ET_ARjr] cell

clone and $3x10^6$ on the CHO-K1[ET_ARjr-flag/Δ5x] cell clone, yet their signaling patterns are indistinguishable. If differential overexpression affected the regulation of the receptors it was not evident from their signaling patterns.

RECEPTOR DESENSITIZATION

Adaptation and desensitization are general terms for cells' ability to decrease responsiveness to prolonged or repeated stimulus. Desensitization can be quantitated by measuring a reduction in signaling induced by a second stimulation relative to a first. Another method for quantitating desensitization is to activate the receptors with a stimulatory pulse, and subsequently record the rate at which the signal decays. Experiments were carried out in cell culture and in *Xenopus* oocytes. Signaling was quantitated in pre-labeled CHO-K1 cell clones by measuring tritiated inositol phosphate levels. Receptor signaling in oocytes was measured using voltage clamping. Desensitization was tested using cells expressing the wild-type receptor. Subsequently ET_ARjr-flag and ET_ARjr-flag/Δ5x constructs were compared in cell culture and Xenopus expression systems to test the hypothesis that phosphorylation of the receptor regulates desensitization.

DESENSITIZATION IN CELL CULTURE

Desensitization was measured in the transfected CHO-K1 cells. The first aspect investigated was the diminished response of receptors to ligand following a prior stimulation event. The responses to each dose of ET-1 were tested by independently measuring the cellular signaling that resulted from each. Cells were exposed to ET-1 for fifteen minutes, followed by washes of various time, and then restimulated. Desensitization in this scenario is the percent reduction between responses to the first and second stimuli.

Cells expressing the wild-type ET_ARjr construct were tested to verify that the cloned receptor desensitized. The relative response of these cells to a second dose of ET-1, one hour after the first, was 28%. This indicated that the wild-type construct underwent desensitization and would be suitable as a template with which mutants could be compared.

CHO-K1[ET_ARjr-flag] and CHO-K1[ET_ARjr-flag/Δ5x] cells were tested for desensitization. Comparison of these two constructs was intended to assess the role of the five potentially phosphorylated serines in desensitization. The response elicited by each receptor to the first dose was arbitrarily called 100%. Both receptor constructs displayed profound desensitization when exposed to ET-1. The response of both receptor types to a second dose of ET-1 dropped quickly, reaching a minimum responsiveness by one hour (see figure 13). Subtracting out the residual levels of IPs from the first dose of ET-1 (see fig14), it was determined that the second dose elicited a 12.6% response in CHO-K1[ET_ARjr-flag] cells, and a 17.8% response in CHO-K1[ET_ARjr-flag/Δ5x] cells (see table 4).

	ET_ARjr-flag	ET_ARjr-flag/Δ5x
% of maximum IPs remaining after 60' wash (#1)	6.0%	22.5%
% of maximum IPs elicited from second dose of ET-1 (#2)	18.6%	40.3%
IPs resulting from second dose of ET-1 (2-1=#3)	12.6%	17.8%

Table 4. Percent of maximum stimulation measured relative to naive cells exposed to a single stimulatory dose of $1x10^{-8}$M ET-1 for fifteen minutes. Results in #1 and #2 both represent cells stimulated with a dose of $1x10^{-8}$M ET-1 for fifteen minutes, and then washed in media for 60 minutes. Following this wash cells in #1 were exposed to 10mM LiCl for fifteen minutes, and cells from #2 were exposed to a second dose of $1x10^{-8}$M ET-1, and 10mM LiCl for fifteen minutes.

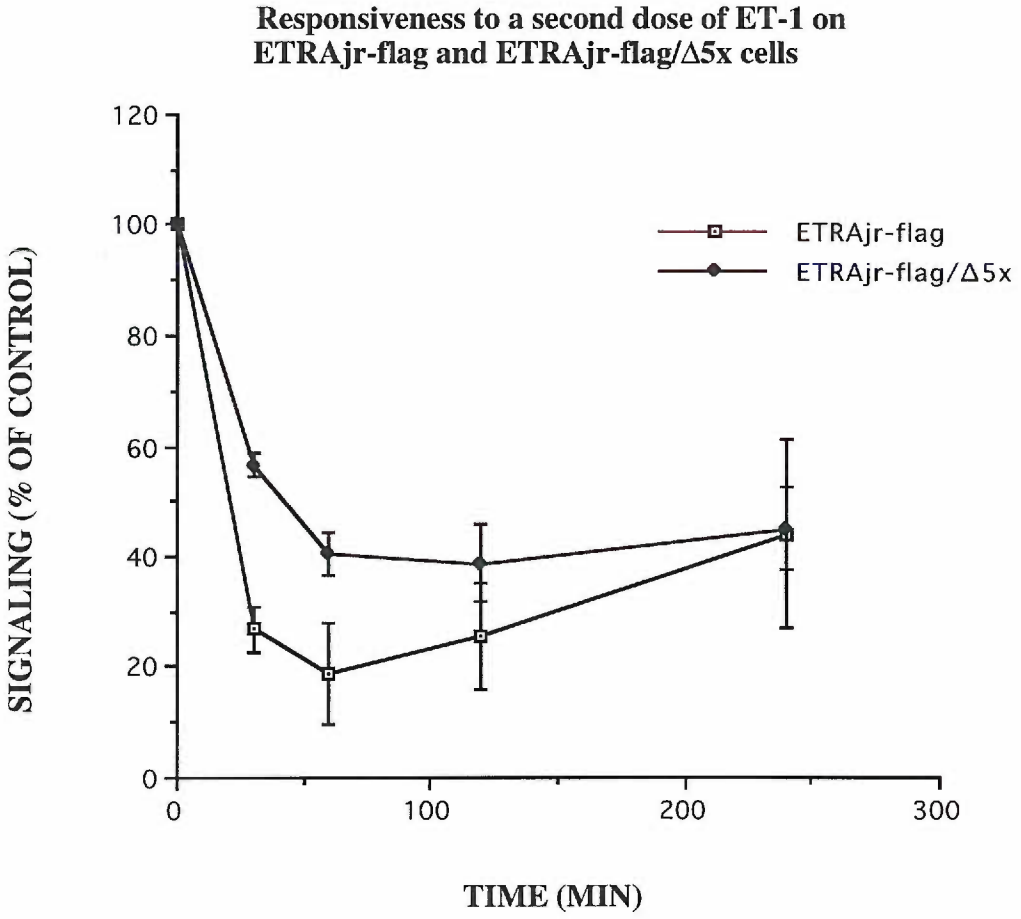


Figure 13. CHO-K1 cell clones expressing either ETARjr-flag or ETARjr-flag/Δ5x were stimulated with 10^{-8}M ET-1 for fifteen minutes, washed for specified amount of time, and then restimulated again with 10^{-8}M ET-1 for fifteen minutes, this time in the presence of 100mM LiCl. At the end of the second stimulation, cells were lysed and assayed for inositol phosphates as described in Materials and Methods. All values are reported relative to IP levels measured for cells previously unstimulated.

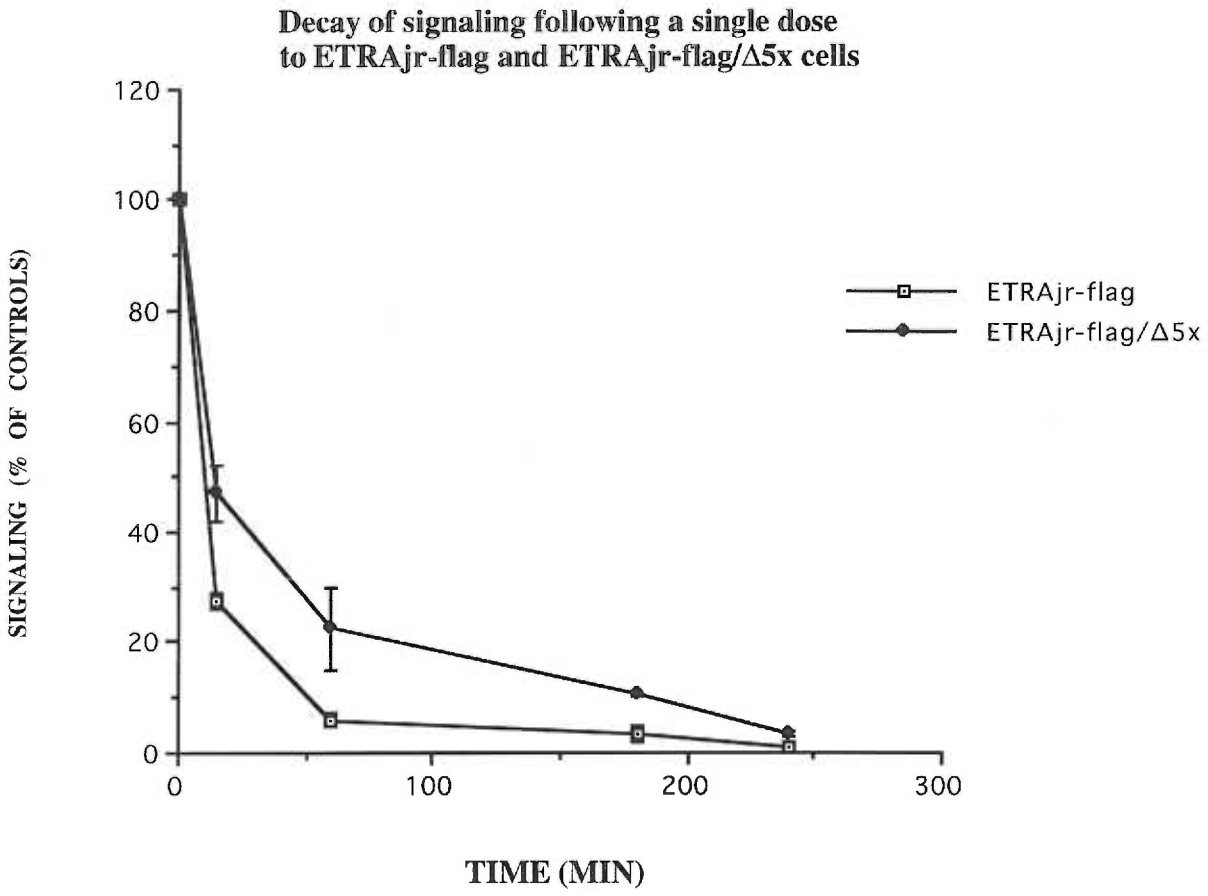


Figure 14. Decay of cellular signal over time following a pulse of stimulation. CHO-K1 cells expressing either ETARjr-flag and ETARjr-flagΔ5x were exposed to a single fifteen minute dose of 10^{-8}M ET-1 and 100mM LiCl, and then incubated for specified lengths of time before being lysed and assayed for residual IP levels, as described in Materials and Methods.

This result does not uphold the hypothesis being tested. Cells lacking serines thought to be phosphorylated were expected to demonstrate little or no desensitization.

The rate at which an activated receptor stops signaling is another aspect of desensitization tested in CHO-K1 cell clones. Cells were stimulated with a pulse of ET-1 and IP levels were assayed at fifteen minute intervals (see figure 15). IP levels in CHO-K1[ET_ARjr-flag] and CHO-K1[ET_ARjr-flag/Δ5x] cells decreased most rapidly over the first 30 minutes following stimulation. The IP levels continued to fall until reaching background levels after four hours of washing. Signaling patterns recorded for both receptors throughout this experiment were similar. This appears to rule out the possibility that phosphorylation at the five sites mutated is responsible for interfering with receptor signaling as a mechanism of desensitization.

DESENSITIZATION IN *XENOPUS* OOCYTES

Desensitization was also measured in the oocyte expression system following a stimulation/wash/stimulation technique similar to the protocol used in cell culture. One minute exposure to $1x10^{-8}$M ET-1 was used for stimulation because it gave a maximal response in earlier experiments. The time between stimuli was 30 minutes; longer washes resulted in increased cell death. Cells were voltage clamped at -60mV, and currents were recorded throughout. Recording chloride ion flow gave a real-time representation of cellular activity.

The oocytes expressing either non-mutant or mutant receptors responded fully to the first dose of ET-1. The current peaked instantly at about 700nAmps and lasted one to two minutes. A second peak in current, superimposed on the tail of the first, reached about 250nAmps at 10 minutes, and returned slowly to baseline. At the end of the 30 minute wash, cells were restimulated. Neither of the receptor constructs responded significantly (see figure 15). These experiments provided evidence that there was no difference between the wild-type and mutant forms of the receptor in desensitization

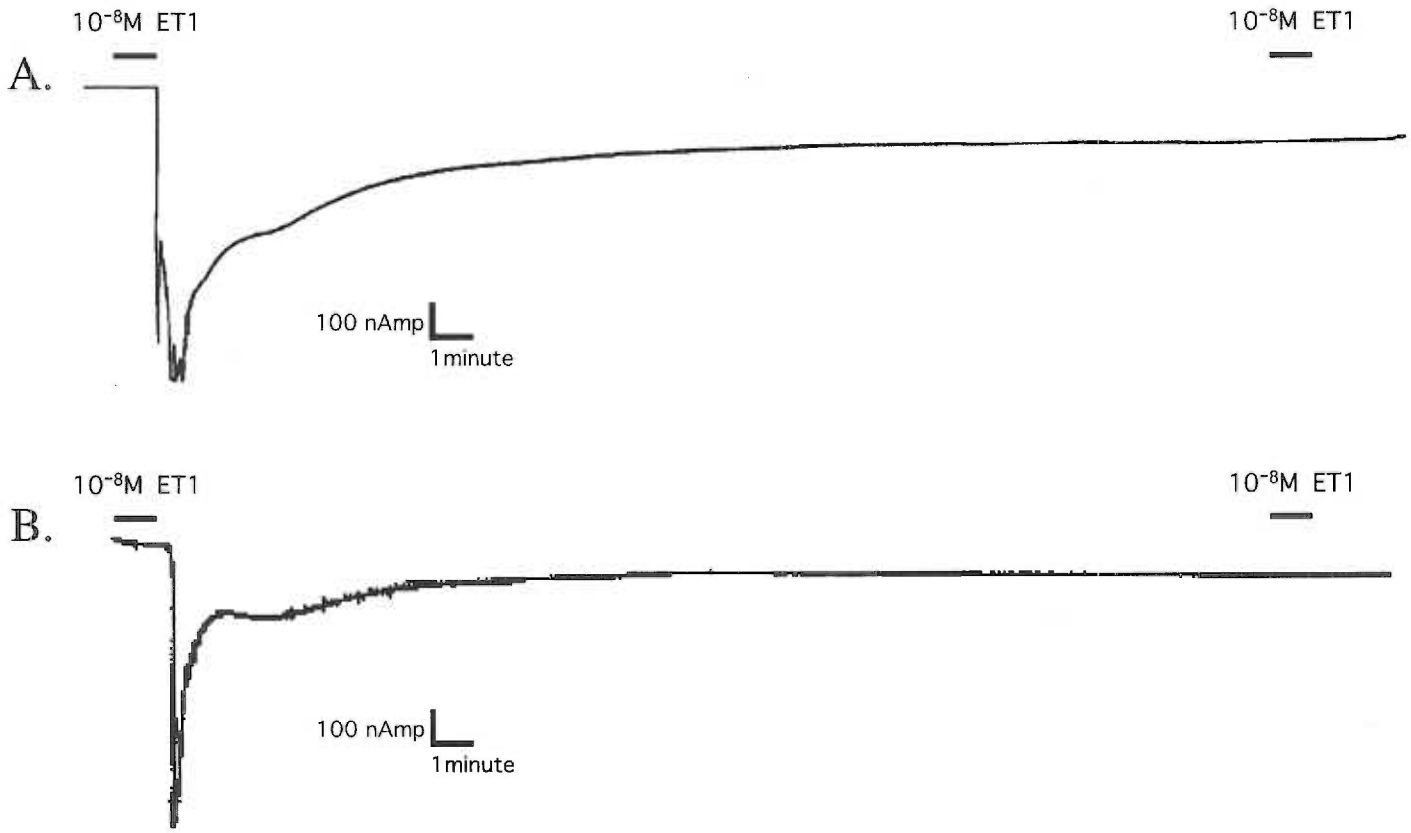


Figure 15a and 15b. Xenopus oocytes expressing A. ETRAjr-flag or B. ETRAjr-flag/Δ5x. Oocytyes injected with *in vitro* transcribed mRNA 3-7 days prior were voltage clamped at -60mV using two electrodes. These clamped cells were then exposed to $1x10^{-8}$M ET-1 for one minute, washed for 30 minutes and then re-exposed to $1x10^{-8}$M ET-1 for one additional minute. Inward current flux is represented as a downward deflection with an intensity represented by the scale markings given.

processes in from oocytes. However, controls were not done that would be necessary to demonstrate that the cell retained its ability to signal during this refractory period, and further that the ETRs were desensitized, and not simply lost.

DISCUSSION

The ET_ARjr desensitization response to ET-1 in oocytes and cell culture is consistent with other studies of the endothelin-A receptor (98, 107). My model of ET_AR desensitization predicted that the ET_ARjr-flag/Δ5x construct would have a signaling pattern distinct from the non-mutant. Possible differences could have included the mutant receptor signaling longer than non-mutants following a pulse of the same duration, or the mutant receptor may have resensitized rapidly or not desensitized at all. None of these differences were seen in comparison of the ET_ARjr-flag and ET_ARjr-flag/Δ5x receptor constructs. In conclusion, the phosphorylation of the receptor at these five serines is not involved in the aspects of desensitization measured in this system.

RECEPTOR INTERNALIZATION

One mechanism that contributes to desensitization is the transport of receptors away from the cell surface. If the net rate of internalization exceeds the rate of externalization, fewer receptors will be exposed on the cell surface. The translocation of receptors to the cell interior makes them inaccessible to ligand and unable to be activated.

IMMUNOFLUORESCENCE

To evaluate whether ETRs are redistributed away from the cell surface following ET-1 stimulation, access of extracellular antibody to the receptor was assayed. Non-permeablized cells were incubated with antibodies directed against the flag epitope. The anti-flag antibodies are made from murine sources, and are commercially available from Kodak. The cells were then incubated with fluorescent labeled antibody that recognizes

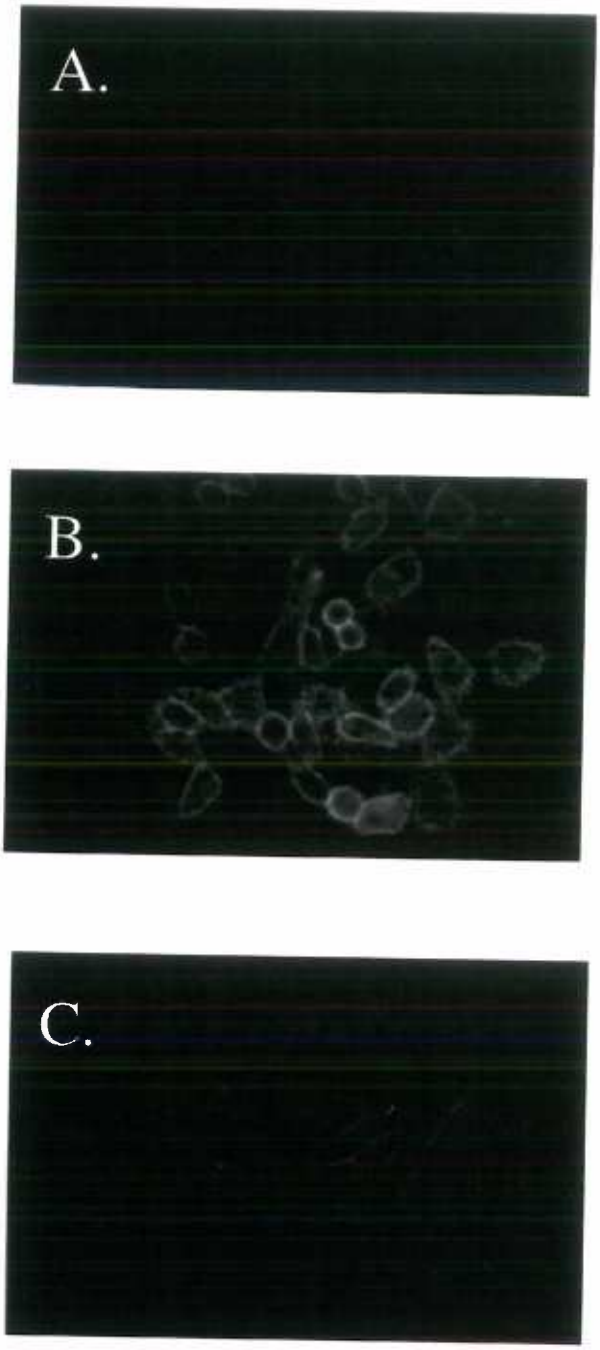
mouse antibodies. Visualizing stained cells on a fluorescent microscope localized flagged proteins to the cell surface.

Comparison of staining patterns in CHO-K1 cells expressing ET_ARjr and ET_ARjr-flag clarified which staining was specific for antibody reacting with flag epitope and which was the result of cross-reaction with native cellular proteins (see figure 16a and 16b). CHO-K1[ET_ARjr-flag] cells were also stained in the absence or presence of excess soluble flag-epitope protein intended to compete against specific binding (see figure 16b and 16c). The fluorescence patterns that resulted from these techniques revealed cell surface staining on cells expressing the flagged receptor, but not on those expressing the non-flagged receptor. The cell surface staining was mostly absent when assayed in the presence of the competitor flag peptide.

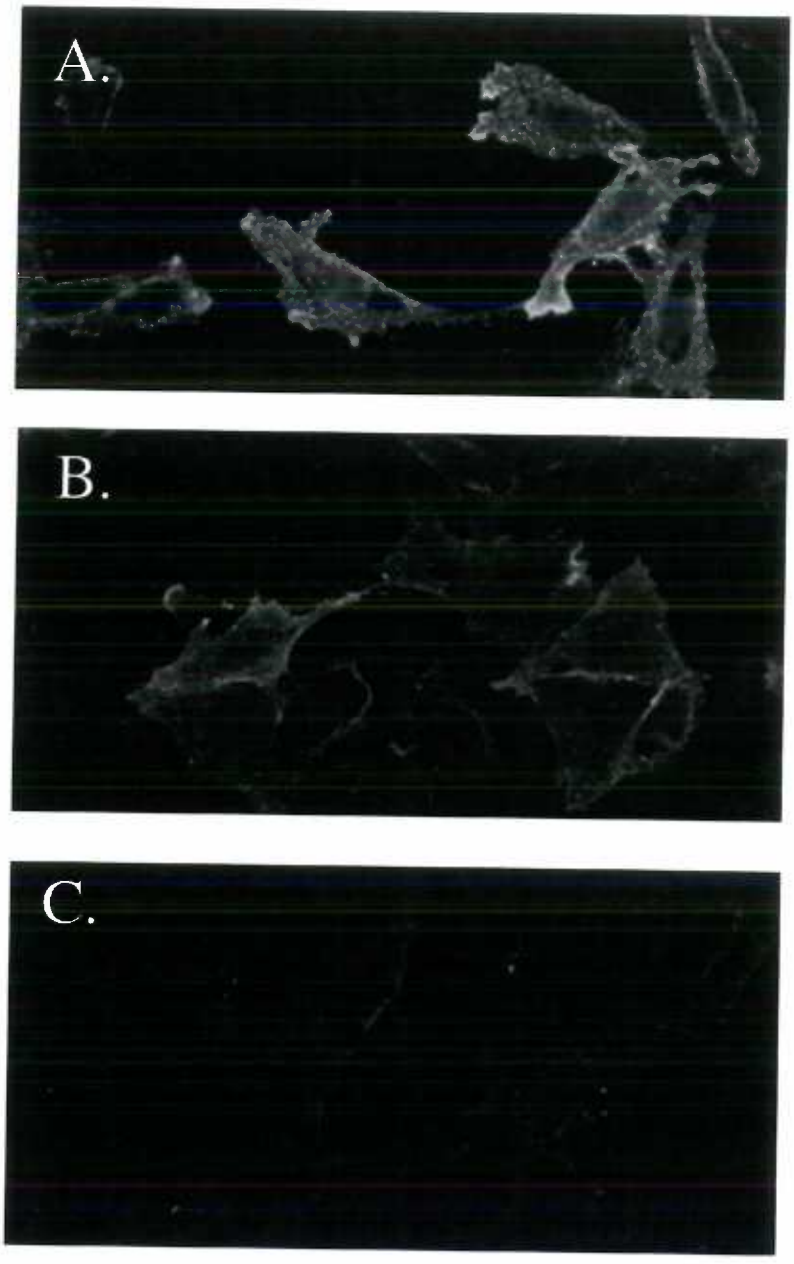
The control experiments in unstimulated cells revealed a distinct cell surface pattern of ET_ARjr-flag. As an assay of receptor internalization, cells were stimulated with ET-1 for various times before staining (see figure 17 and 18). Diminution of staining evidenced that receptors were redistributed away from the cell surface as a result of stimulation with ET-1. The staining patterns before and after stimulation were similar for cells expressing the different receptor constructs. Both receptors appeared to be internalized as a function of time following stimulation with ET-1.

SATURATION AND INTERNALIZATION

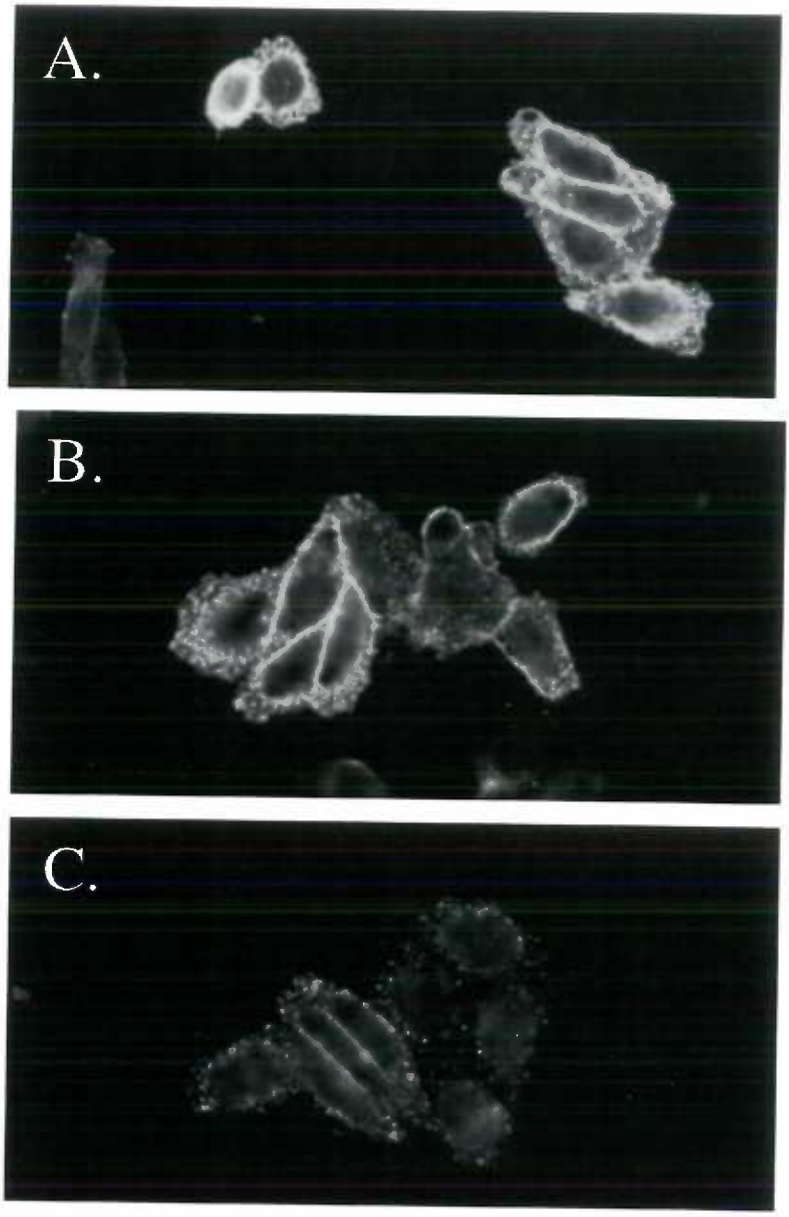
The pH of the environment affects the binding of many receptors to their ligands. Changing the pH influences the receptor-ligand interaction such that binding can be prevented over some pH ranges, and reversed over other ranges. Acidification of media can effectively dissociate ET_AR-ET-1 complexes from the surface of vascular smooth muscle cells (108). Acid wash only removes ligand from the external surfaces of the cell, making it possible to differentiate between surface and internalized ligand.



Figures 16A, 16B and 16C. CHO-K1 cells expressing: A. ETRAjr, B. ETRAjr-flag, or C. ETRAjr-flag, in the presence of 10mg/ml soluble flag peptide, were stained first with M2 anti-flag antibody for 90 minutes and subsequently with goat anti-mouse FITC antibody for 60 minutes. All three panels represent photos taken at identical F-stops and shutter speeds.



Figures 17A, 17B and 17C. CHO-K1 cells expressing ETRAjr-flag stimulated with $1x10^{-8}$M ET-1for: A. 0 minutes, B. 30 minutes, or C. 60 minutes. Cells were then stained with M2 anti-flag antibody for 90 minutes and subsequently with goat anti-mouse FITC antibody for 60 minutes. All three panels represent photos taken at identical F-stops and shutter speeds.



Figures 18A, 18B and 18C. CHO-K1 cells expressing ETRAjr-flag/Δ5x stimulated with $1x10^{-8}$M ET-1for: A. 0 minutes, B. 30 minutes, or C. 60 minutes. Cells were then stained with M2 anti-flag antibody for 90 minutes and subsequently with goat anti-mouse FITC antibody for 60 minutes. All three panels represent photos taken at identical F-stops and shutter speeds.

Acid treatment does not damage the ET receptor or the CHO-K1 cell. This was demonstrated in cells expressing ET_ARjr-flag constructs by prewashing untreated cells with acid, and subsequently binding ^{125}I ET-1. Comparing the amount of ET-1 bound to acid washed cells and to unwashed cells revealed that acid treatment resulted in only a minor reduction (~10%) in total binding (data not shown).

Temperature has profound effects on internalization. Incubation at 4°C inhibits the internalization of many different receptor-ligand complexes (108, 140)]. Resink reported that 4°C treatment inhibits internalization without effecting the receptor's ability to recover internalization processes when returned to 37°C (108).

The effect of chilling cells to 4°C was assessed by measuring the amount of ligand associated with the cell surface incubation at 4°C in the presence of ^{125}I ET-1. After three hours of binding, cells were acid washed, which revealed that 90-94% of the bound ligand remained sensitive to acid, indicating it was located on the cell surface (data not shown). The control experiments revealed that ligand could be bound and that the receptor-ligand complex remained at the cell surface at 4°C. These experiments also revealed that the surface-bound ligand could be removed with an acid wash. This information was used to determine the rate of receptor saturation and internalization.

ACID WASH OF SURFACE BOUND LIGAND

Internalization was assayed using a technique involving saturation of binding sites on the cell surface. After saturation, the fraction of receptors internalized and on the cell surface was followed over time. The saturation was carried out at 4°C to inhibit internalization during this period. Subsequent internalization was achieved by transferring the cells to 37°C. The cell surface and internalized ligand were measured as fraction of total ligand bound. Surface ET-1 was released and quantitated using the acid wash technique. Following the acid wash, cells were lysed to determine the acid-insensitive, or internalized, amounts of labeled ET-1.

These experiments were designed to compare the internalization kinetics of the receptor constructs with and without potential phosphorylation sites. Surface receptors of CHO-K1 cells expressing ET_ARjr-flag or ET_ARjr-flag/Δ5x bound by ET-1 internalized rapidly (see figure 19). A plateau of 40-50% of bound receptor internalization is reached after 30-60 minutes. Although the rate is similar for the two constructs, neither one internalized all of the bound receptors. Internalization of ET_AR is cell type dependent, which may explain the incomplete internalization of the ET_ARjr-flag and ET_ARjr-flag/Δ5x constructs in CHO-k1 cells. Vascular smooth muscle cells internalize a maximum of about 70% of their cell surface ET receptors, whereas hepatocytes reach a maximum internalization as high as 93% (105, 108).

SATURATION OF CELL SURFACE RECEPTORS

The rate at which receptors become bound and internalized can influence the response to a given stimulus. The time required for cell surface ET_ARs to be saturated with ET-1 is about one hour in vascular smooth muscle cells (110). In striking contrast, Marsault calculated the theoretical saturation of ET_AR on aortic myocytes to be 95% after a two minute incubation (141). The difference between the theoretical and experimental times necessary for saturation imply that some factor has not been taken in to account in the theoretical model.

Saturation rate was measured by prebinding cells with unlabeled ET-1 at 37°C for varying times, followed by saturation of remaining sites with ^{125}I ET-1 at 4°C . The fraction of maximum ^{125}I ET-1 binding present at any time represented the degree to which the cell surface receptors were occupied. Mutant and non-mutant ET_ARs were assayed for their saturation rates (see figure 20). Analysis of these clones showed that 70-85% of sites capable of binding were no longer accessible to ligand after a one hour incubation with ET-1. Comparison of the ET_ARjr-flag and ET_ARjr-flag/Δ5x receptors revealed differences in saturation rate. The mutant receptors were saturated more rapidly

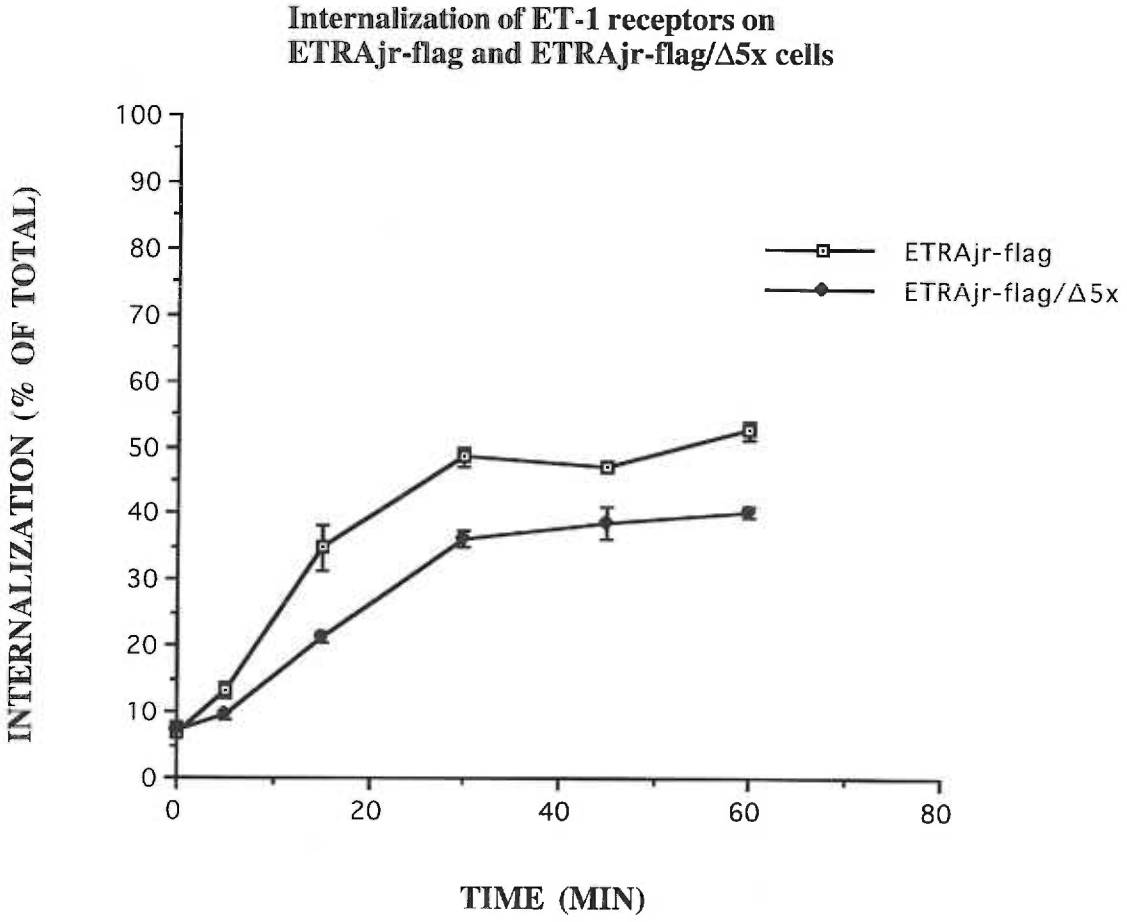


Figure 19. Cells expressing ETARjr-flag and ETARjr-flag/Δ5x recepors were incubated with radiolabeled ET-1 for three hours at 4°C, and then moved to 37°C to permit internalization. At specified times cells were acid washed to remove cell surface receptors, and then the cells were lysed. Percent internalization was calculated as follows: (acid sensitive)/(acid sensitive + acid resistant).

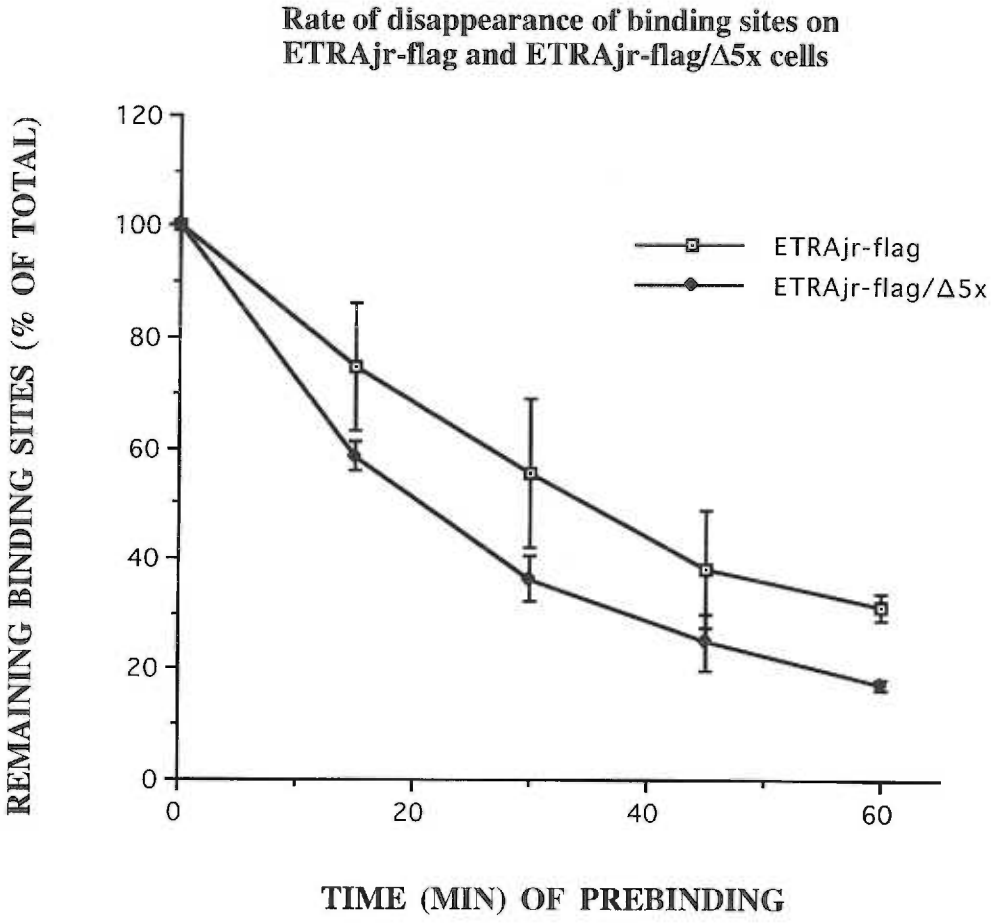


Figure 20. Saturation rates were measured for ETARjr-flag and ETARjr-flag/Δ5x expressing cells. Binding sites were determined by incubating the cells at 37°C with ET-1 for the designated period of time, and then transferring them to 4°C for three hours to saturate the remaining sites with radiolabeled ET-1. The 100% value was measured on cells that were exposed to the radiolabeled ET-1 alone at 4°C.

than the non-mutant receptors, which is contrary to the prediction that the loss of phosphorylation sites negatively effects desensitization. Maximal saturation was reached near one hour which is consistent with most published reports (108, 110).

Binding study results, when combined with the immunofluorescence data, suggest that the ET_ARjr constructs are internalized in a ligand-dependent manner, and that internalization is inhibited in cells chilled to 4°C. It is clear from these experiments that mutation of phosphorylation sites does not prevent internalization. One possible explanation for incomplete internalization is that the cell clones may express high enough levels of receptors that cellular processes involved in internalization are unable to complete the task. This explanation is unlikely, however, because if internalization mechanisms were overwhelmed the cells expressing the mutant receptor would not have internalized twice the number of receptors as the non-mutant expressing cells.

ET-1 OFF-RATE

The Kd of the ET_AR for ET-1 has consistently been reported to be 100-400 pM (12). Reports of reversible binding of ET-1 to C6 glial cells and irreversible binding to vascular smooth muscle cells suggests variability in the receptor-ligand interactions between cells (110, 142). If bound ET-1 were to dissociate from the receptor at an appreciable rate, the concentration of ligand in subsequent incubations would be greatly affected. To test the off-rate of ET-1 from CHO-K1[ET_AR jr-flag], the cell surface receptors were saturated with labeled ET-1 and the cell culture medium was monitored for labeled ET-1. This was done in the presence of non-radiolabeled competitor to prevent rebinding of labeled ET-1. Nine percent of the surface-associated ET-1 was released into the medium in the first 30 minutes. This increased to 10% by one hour, and to 13% by two hours (data not shown). The results suggest that the majority of receptors

have a very slow dissociation from bound ligand. The sharp initial rise in release could be the result of either a different binding state, or a fraction of ligand which was nonspecifically bound being released more rapidly.

ET_AR EXTERNALIZATION

Internalization is the process that moves receptors from the cell surface to intracellular compartments. Externalization is opposite, transporting receptors from intracellular compartments to the cell surface. The difference in the rates of the two processes determines cell surface density of receptors. If desensitization of ET_AR is accomplished solely by removing the receptor from the cell surface, resensitization should be reflected in the rate of externalization. Different techniques have been reported in the literature to assay the rate of externalization. To test the rate of ETR externalization of hepatocytes, cell surface ET_ARs were saturated with ET-1 to stimulate receptor internalization. The receptor binding capacity slowly recovered, but the phosphoinositide response remained inhibited for an additional 24 hours (105). The presence of receptors insensitive to ET-1 implies that desensitization is regulated independently from internalization and externalization. Contrary to the hepatocyte data, Marsault reported that externalization of ET_AR on aortic myocytes determines the rate of receptor resensitization (141). These data predict that externalization is the mechanism that resensitizes populations of receptors, and therefore determines rate and length of desensitization. The different models of resensitization may be attributable to cell type variation, or distinctions in techniques used.

To measure the rate at which receptors are externalized in CHO-K1[ET_AR jr-flag] and CHO-K1[ET_AR jr-flag/Δ5x] cells, surface receptors were saturated, and then allowed to incubate for varying lengths of time before rebinding with ^{125}I ET-1. The amount of label bound to the cell represents the number of receptors newly available to be bound.

The total ^{125}I ET-1 binding is a combination of off-rate and externalization. The off-rate, as described above, is very slow, and because the wash period was done in the absence of excess cold competitor the rate was expected to be negligible. This experiment revealed no externalization could be measured for either cell type under the conditions described. It is possible that externalization occurred at a rate too slow to register in these experiments. It is also possible that the externalization process is inhibited in these cells following activation. Inhibition of externalization would prevent the return of cell surface receptors, and could be responsible for prolonged desensitization. Slow externalization would best explain Ghandi's model of slow recovery of receptor numbers, preceding recovery of signaling (105). The slow rate of receptor recovery is very different from the rapid recovery reported for aortic myocytes (141). It is possible that the difference in maximum receptor density on the aortic myocytes and on CHO-K1[ET_ARjr-flag] and CHO-K1[ET_ARjr-flag/Δ5x] cells affected the measurable externalization rate. If the CHO-K1 cell clones were externalizing the same number of receptors as reported for the myocytes, it would represent less than one percent of the total binding.

IMMUNOPRECIPITATION

The hypothesis tested in this thesis is that phosphorylation at specific sites on the ET_AR is involved in desensitization. Characterizing receptor constructs with and without the potentially phosphorylated sites should determine their effect on receptor control. Of course the first assumption made in the hypothesis is that the receptor is phosphorylated; hence, it would be informative to show that ET_AR is a phosphoprotein, and that phosphorylation is modulated by ET-1 stimulation. Several reports refer to the existence of putative phosphorylation sites, but none provide the necessary supporting physical evidence. The presence of consensus sequences, and the reports of similar GPCRs being phosphorylated is circumstantial evidence for such an event. The intention, through the

use of immunoprecipitation techniques, was to prove that the receptor is phosphorylated. Unfortunately, immunoprecipitation of flagged proteins did not occur. The only proteins that precipitated were either non-specific or cross reacting.

Immunofluorescence studies described above revealed that the flag epitope is present on the endothelin receptor, and that it is recognized by the M2 anti-flag antibody (see figure 16). It is not clear why the M2 antibody can recognize epitope presented on the cell surface, as seen in immunofluorescence, and yet not work in immunoprecipitation. It is possible that disruption of the plasma membrane, caused by lysis of cells in preparation of precipitation, results in a change in conformation making the epitope inaccessible to antibody. Several of the conditions described above represent attempts to address this possibility. One way around this problem would be to use an antibody directed at a portion of the receptor itself. Antibodies directed against the ET_AR have been described in the literature, but are not yet available commercially (144, 145).

CHAPTER 4
SUMMARY AND CONCLUSIONS

The work described in this thesis was undertaken to gain better insight into the processes involved in desensitization of the endothelin-A receptor. Desensitization is important in regulating normal physiology at the cellular level. It is also likely that any significant changes in the control of desensitization would result in a disease state. An experimental system was constructed to test the model of ET_AR desensitization. The model consisted of a two stage process. The first stage is receptor phosphorylation, which results from cellular signaling in response to ligand binding. The second step is internalization. Following ligand binding the cell is activated, the receptor is phosphorylated, and the receptor-ligand complex is endocytosed. The net effect of these two steps is to restrain cellular activation in response to repeated or prolonged stimulation with ET.

Construction of the experimental system started with the cloning of a cDNA coding the human ET_AR using polymerase chain reaction techniques. The flag epitope was then inserted into the amino terminal portion of the receptor. The intention was to manipulate the protein structure so that it would be antigenic for commercial antibodies and therefore useful for immunoprecipitation and immunofluorescence techniques. Site directed mutagenesis was used to insert the 24 base pairs coding for the flag epitope. This technique was also used to mutate specific (cytoplasmic) serines that fit the consensus amino acid sequence for potential phosphorylation sites. The flagged receptor was used as a template for altering the serines so that the resulting mutants would be recognized by the available antibodies. The wild-type (WT), flag, and flag/mutant receptors were expressed and tested in two different experimental systems. *In vitro* transcribed mRNA coding for the flag and flag/mutant receptors was injected into

Xenopus oocytes for electrophysiologic analysis. Also, the wild-type, flag and flag/mutant constructs were stably transfected into a Chinese hamster ovary cell line.

Voltage clamping of Xenopus oocytes provided a means to measure cellular responses to stimuli over very short time periods. Oocytes expressing either flag or flag/mutant receptors responded similarly to a single dose of ET-1. The activation of the endothelin receptor stimulated the release of intracellular stores of calcium, which was followed by an influx of extracellular calcium. The response elicited from both receptor types lasted for 20-30 minutes (see figure 11).

When the receptors expressed in oocytes were tested for desensitization, they were found to be profoundly affected (see figure 15). The two receptors (flag and flag/mutant) responded strongly to the first ET-1 dose, and neither one responded to the second dose. The similarity of patterns seen for the different receptors is contrary to predictions based on the model of desensitization. These results are supported by recent reports in which deletion mutants of the endothelin-A receptor were expressed in *Xenopus* oocytes (99, 100). This work involved truncating the receptor 36 amino acids proximal to the carboxyl tail, thereby eliminating four of the five consensus sequences mutated in my construct. Comparison of the truncated receptor to the wild-type receptor revealed that both constructs were desensitized to the same degree. The receptors were also monitored for resensitization to ET-1 in these experiments. The times necessary for oocytes to recover sensitivity to the hormone were the same whether they were expressing the truncated or the full length receptors (100).

Binding studies were performed on the stable transfectants in culture to characterize the binding affinities of the receptor constructs for endothelin-1. Binding studies also made it possible to determine the number of receptors expressed on the surface of each cell clone. Scatchard analysis of the binding data revealed that insertion

of the flag epitope and mutation of selected serines had no effect on the affinities of the receptors for endothelin-1 (see figure 9). Analysis included a vascular smooth muscle cell line as a control for a naturally expressed endothelin-A receptor. The Kds calculated from the Scatchard plots were consistent with published reports (12). The number of endothelin binding sites on each cell was calculated from the Scatchard plots (see figure 9). Cell clones for the three constructs expressed extremely high levels of receptor numbers, which varied by as much as five fold between clones. At the same time, the number of receptors per cell calculated for the vascular smooth muscle cells was identical to that in published reports. Flag/mutant receptor CHO-K1 cell clones expressed the highest density of receptors, reaching more than 200 times the expression levels of the control amooth muscle cells. The overexpression of these receptors could potentially overwhelm the cellular processes involved in desensitization. One approach to eliminating the potentially complicating effects of high receptor density would be to repeat the transfection of receptor constructs, selecting cell clones that express receptors at levels closer to the density found on cells that express the receptor naturally.

Levels of inositol phosphate were used as an indicator of receptor activity in cell culture experiments. Dose-response comparisons of mutant and non-mutant constructs showed that both receptors respond similarly to ET-1 over the entire range of concentrations tested (see figure 10). The concentration eliciting a half maximal response was the same for the two receptors, and was in agreement with published reports.

Inositol phosphate levels were also used to assay the rate at which cellular signaling declines after a transient exposure to ET-1. Following a limited period of activation, receptor signaling decreased rapidly, indicating that a cellular mechanism involved in signaling was being actively inhibited (see figure 14). The signals produced when flag and flag/mutant receptors were activated decayed rapidly, but slightly more rapidly for the non-mutant. Levels of inositol phosphates in cells expressing each of the

receptors decreased most rapidly over the first hour, and by the end of four hours had returned to preactivation levels. Studies of off-rates revealed that the decrease in signaling could not be attributed to ligand dissociating from the receptor. The rate of decay was slower in cells expressing the flag/mutant receptor compared to those expressing the flag. It is not likely that the difference between clones was the result of the mutations. Small variations are likely between clonal populations of cells. A report of similar measurements made on primary cultures of cardiac myocytes (107) revealed residual levels of inositol phosphates slightly higher than seen in the flag/mutant.

Inositol phosphate levels were also used to measure receptor desensitization. Experiments revealed that mutant and non-mutant receptors were profoundly desensitized in response to stimulation with ET-1 (see figure 13). The response to the second dose, relative to the response to the first dose, was greatly diminished. When comparison of residual inositol phosphate levels with second dose totals, both receptors were only responding a fraction of the intensity induced by the primary stimulus. The similarity between clones is contrary to the hypothesis that the mutated serines are involved in desensitization. The patterns of desensitization for both receptors are similar to the patterns seen in a variety of cells and tissues naturally expressing the endothelin-A receptor (42, 107, 111).

Radioligand binding studies were designed to assess the role of ligand binding and internalization in receptor desensitization. The first experiments revealed that flag and flag/mutant receptors on the cell surface require nearly one hour of exposure to ET-1 to reach saturation at 37°C (see figure 20.). The fraction of unbound sites remaining after incubation with ET-1 was used to calculate the time course of saturation. Quantification of saturation involved sequential incubations with unlabeled ET-1 followed at various times by radiolabeled ET-1. Consistent with the result obtained from this experiment,

other cells studied under similar conditions required about an hour to fully saturate their cell surface endothelin receptors (108, 110, 141).

Any significant dissociation of receptor-ligand complex during the second binding period would decrease the specific activity of the label, and could give deceptively low saturation fractions. To eliminate this possibility off-rate was measured. Experiments revealed a slow rate of release of ET-1 from cells expressing the flag receptor. Therefore, ET-1 released from the cell surface did not contribute significantly to the measurement of saturation. The measurement of a slow off-rate has been reported for the endothelin receptor in other cell types as well (101, 133).

Additional radioligand binding studies were carried out to measure the rate of receptor internalization. After activation by ET-1 binding, flag and flag/mutant receptors both internalize rapidly, reaching a plateau between 30 minutes and one hour (see figure 19). Internalization was measured as the fraction of ligand bound receptors that were insensitive to an acid wash at a given time. Both receptor constructs tested internalized a substantial number of their cell surface receptors in response to ligand binding. The flag/mutant expressing cells, however, internalized about a 10% smaller fraction of the total number of receptors when compared to the flag expressing cells. This difference may reflect a difference in regulation resulting from the mutated serines. It may also be the result of higher receptor density on the surface of cells expressing the mutant. It is unlikely that the cellular mechanisms were saturated. Even though the flag cells internalized a 10% larger fraction of their receptors the actual number or receptors moved was half as many as in the mutant expressing cells.

Internalization was also assessed using immunofluorescence techniques. Stimulating mutant and non-mutant receptors for varying times prior to staining, revealed a time-dependent loss of cell surface fluorescence (see figures 17 and 18). This analysis was not quantitative, but gave visual results that paralleled the binding data.

Immunoprecipitation was used in order to isolate receptors expressing a flag epitope. This technique is often useful in determining the phosphorylation state of receptor constructs in various activation states. The technique was unsuccessful at isolating metabolically labeled receptors. Anti-flag antibodies derived from two independent systems were both tested to no avail. One of the antibody preparations had worked previously when tested in immunofluorescence studies, but did not work in any of the attempts at immunoprecipitation. Numerous different conditions were used but none enabled precipitation of any protein from the transfected cells that was not also present in non-transfected cells. A likely solution to this problem will be to use antibodies directed against the extracellular portion of the endothelin receptor, rather than an artificially engineered epitope. Anti-endothelin-A receptor antibodies have been produced, but are not yet commercially available (143, 144).

The endothelin-A receptor was cloned and determined to be free of mutations. Expression of this receptor in tissue culture cells provided evidence that the receptor binds ligand and signals in a manner consistent with published literature. The purpose of this body of work was to test the hypothesis that desensitization is regulated through phosphorylation of the receptor at serines that fit a consensus sequence motif for phosphorylation by protein kinase C. To test this hypothesis the DNA sequence of the receptor was mutated to alter five potentially phosphorylated serine residues, substituting alanines at these positions. Analysis of the mutant receptor revealed that replacement of

the serines had no effect on receptor desensitization. The result is surprising and significant. A review of the current literature predicts involvement of these serines in regulation of receptor activity. This is apparently not the case. Clearly, an alternate mechanism for mediating internalization and desensitization remains to be discovered.

REFERENCES

1. Furchgott RF&Z J.V.1980. The obligatory role fo endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature;288:373-376.

2. Moncada S, Vane J.1979. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromoxane A_2 and prostacyclin. Pharmacol. Rev.;30:293-331.

3. Plamer RMJ, Ferrige AG, Monacada S.1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factro. Nature;327(524-526).

4. Yanagisawa M, Masaki T.1989. Endothelin, a novel endothelium-derived peptide. Biochemical Pharmacology;38(12):1877-1883.

5. O'Brien R, Robbins R, McMurtry I.1987. Endothelial cells in culture produce a vasoconstrictor substance. J. Cell Physiol.;132:263-270.

6. Hickey K, Rubanyi G, Paul R, Highsmith R.1985. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. Am. J. Physiol.;248:C550-556.

7. Gillespie M, Owasoyo J, McMurtry I, O'Brrien R.1986. Sustained coronary vasoconstriction provoked by a peptidergic substance released from endothelial cells in culture. J. Pharmacol. Exp. Ther.;236:339-343.

8. Yanagisawa M, Kurihara H, Kimura S, et al.1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells [see comments]. Nature;332(6163):411-5.

9. Fabbrini M, Valsasina B, Nitti G, Benatti L, Vitale A.1991. The signal peptide of human preproendothelin-1. FEBS;286(1,2):91-94.

10. Kimura S, Kasaya Y, Sawamura T, et al.1989. Conversion of big endothelin-1 to 21-residue endothelin-1 is essential for expression of full vasoconstrictor activity: Structure-activity relatioships of big endothelin-1. J. Cardiovasc. Pharmacol.;13:S5-S7.

11. Waxman L, Doshi KP, Gaul SL, Wang S, Bednar RA, Stern AM.1994. Identification and characterization of endothelin converting activity from EAHY 926 cells: evidence for the physiologically relevant human enzyme. Archives of Biochemistry and Biophysics;308(1):240-53.

12. Sokolovsky M.1992. Endothelins and sarafotoxins: physiological regulation, receptor subtypes and transmembrane signaling. Pharmacology and Therapeutics;54(2):129-49.

13. Huggins JP Pelton, J.T., and Miller, R.C.1993. The structure and specificity of endothelin receptors: Their importnace in physiology and medicine. Pharmac. Ther.;59:55-123.

14. Cozza E, Gomez-Sanchez C, Foecking MF, Chiou S.1989. Endothelin binding to cultured calf adrenal zona glomerulosa cells and stimulation of aldosterone secretion. J. Clin. Invest.;84:1032-1035.

15. Stojilkovic S, Iida T, Merelli F, Catt K.1991. Calcium signaling and secretory responses in endothelin-stimulated anterior pituitary cells. Molecular Pharmacology;39(6):762-770.

16. Muldoon L, Pribnow D, Rodland K, Magun B.1990. Entothelin-1 stimulates DNA synthesis and anchorage-independent growth of Rat-1 fibroblasts through a protein kinase C-dependent mechanism. Cell Regulation;1:379-390.

17. Suzuki T Hoshi, H. & Mitsui, Y.1990. Endothelin stimulates hypertrophy and contractility of neonatal rat cardiac myocytes in serum-free medium. FEBS Lett.;268:149-151.

18. Hirata Y, Takagi Y, Fukuda Y, Marumo F.1989. Endothelin is a potent mitogen for rat vascular smooth muscle cells. Atherosclerosis;78(2-3):225-228.

19. Leppaluoto J, Ruskoaho H.1992. Endothelin peptides: biological activities, cellular signalling and clinical significance. Annals of Medicine;24(3):153-61.

20. Weitzberg E, Ahlborg G, Lundberg J.1991. Long-lasting vasoconstriction and efficient regional exreaction of endothelin-1 in human splanchnic and renal tissues. Biochemical and Biophysical Research Communications;180(3):1298-1303.

21. Inoue A, Yanagisawa M, Kimura S, et al.1989. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proceedings of the National Academy of Sciences of the USA;86(8):2863-7.

22. Masaki T, Kimura S, Yanagisawa M, Goto K.1991. Molecular and cellular mechanism of endothelin regulation: implications for vascular function. Circulation;84:1457-1468.

23. Masaki T, Yanagisawa M, Goto K, Kimura S, Takuwa Y. Cardiovascular significance of endothelin. In: Rubanyi GM, ed. Cardiovascular significance of endothelium-derived vasoactive factors. Mount Kisco, Ny: Futura Publishing Co., Inc.,, 1991: 65-81.

24. Rubanyi GaLPB.1991. Endothelins. FASEB Journal;5:2713-2720.

25. Giaid A Gibson, S.J., Ibrahim, N.B.N., Legon, S., Bloom, S.R., Yanagisawa, M., Masaki, T., Varndell, I.M., and Polak, J.M.1989. Endothelin 1, an endothelium-derived peptide, is expressed in neurons of the human spinal cord and dorsal root ganglia. Proc. Natl. Acad. Sci. USA;86:7634-7638.

26. Bloch KD Hong, C.C., Eddy, R.L., Shows, T.B. & Quertermous, T.1991. cDNA cloning and Chromosomal Assignment of the Endothelin 2 Gene: Vasoactive Intestinal Contractor Peptide is Rat Endothelin 2. Genomics;10:236-242.

27. Saida K Y. Mitsui, & N. Ishida.1989. A novel peptide, Vasoactive Intestinal Contractor, of a new (endothelin) peptide family. The Journal of Biological Chemistry;264(25):14613-14616.

28. Lerman A, Edwards B, Hallett J, Heublein D, Sandberg S, Burnett J Jr.1991. Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. The New England Journal of Medicine;325(14):997-1001.

29. Kon V, Badr K.1991. Biological actions and pathophysiologic significance of endothelin in kidney. Kidney International;40(1):1-12.

30. Omland T, Lie R, Aakvaag A, Aarsland T, Dickstein K.1994. Plasma endothelin determinations as a prognostic indicator of 1-year mortality after acute myocardial infarction. Circulation;89(4):1573-1579.

31. Goto M, Takei Y, Kawano S, et al.1994. Endothelin-1 is involved in the pathogenesis of ischemia/reperfusion liver injury by hepatic microcirculatory disturbances. Hepatology;19(3):675-681.

32. Clozel M, Breu V, Burri K, et al.1993. Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. Nature;365:759-761.

33. Kondo S, Morita T.1993. Effect of benign prostatic hypertrophy on the endothelin-1 receptor density in human urinary bladder and prostate. Japanese Journal of Urology [Japanese];84(10):1821-1827.

34. Kon V, Yoshioka T, Fogo A, Ichikawa I.1989. Glomerular actions of endothelin in vivo. Journal of Clinical Investigation;83(5):1762-1767.

35. Liu J, Casley D, Nayler W.1989. Ischaemia causes externalization of endothelin-1 binding sites in rat cardiac membranes. Biochem. Biophys. Res. Commun.;164:1220-1225.

36. Kloog Y, Bousso-Mittler D, Bdolah A, Sokolovsky M.1989. Three apparent receptor subtypes for the endothelin/sarafotoxin family. FEBS Letters;253(199-202).

37. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S.1990. Cloning and expression of a cDNA encoding an endothelin receptor [see comments]. Nature;348(6303):730-2.

38. Hosoda, Nakao, Arai, et al.1991. Cloning and expression of human endothelin-1 receptor cDNA. FEBS LETTERS;287(1,2):23-26.

39. Cyr C, Huebner K, Druck T, Kris R.1991. Cloning and chromosomal localization of a human endothelin ETA receptor. Biochemical and Biophysical Research Communications;181(1):184-90.

40. Adachi M, Yang YY, Furuichi Y, Miyamoto C.1991. Cloning and characterization of cDNA encoding human A-type endothelin receptor. Biochemical and Biophysical Research Communications;180(3):1265-72.

41. Elshourbagy NA, Korman DR, Wu HL, et al.1993. Molecular characterization and regulation of the human endothelin receptors. Journal of Biological Chemistry;268(6):3873-9.

42. Lin HY, Kaji EH, Winkel GK, Ives HE, Lodish HF.1991. Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. Proceedings of the National Academy of Sciences of the Usa;88(8):3185-9.

43. Sakurai T, Yanagisawa M, Takuwa Y, et al.1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor [see comments]. Nature;348(6303):732-5.

44. Ogawa Y, Nakao K, Arai H, et al.1991. Molecular cloning of a non-isopeptide-selective human endothelin receptor. Biochemical and Biophysical Research Communications;178(1):248-55.

45. Nakamuta M, Takayanagi R, Sakai Y, et al.1991. Cloning and sequence analysis of a cDNA encoding human non-selective type of endothelin receptor. Biochemical and Biophysical Research Communications;177(1):34-9.

46. Sakamoto A, Yanagisawa M, Sakurai T, Takuwa Y, Yanagisawa H, Masaki T.1991. Cloning and functional expression of human cDNA for the ETB endothelin receptor. Biochemical and Biophysical Research Communications;178(2):656-63.

47. Karne S, Jayawickreme CK, Lerner MR.1993. Cloning and Characterization of an Endothelin-3 Specific Receptor (ETcReceptor) from *Xenopus laevis* Dermal Melanophores. The Journal of Biological Chemistry;268(25):19126-19133.

48. Emori T, Hirata Y, Marumo F.1990. Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action. FEBS Lett.;263:261-264.

49. Berridge M.1993. Inositol trisphosphate and calcium signalling. Nature;361:315-325.

50. Chuang D-M.1989. Neurotransmitter receptors and phosphoinositide turnover. Ann. Rev. Pharmacol Toxicol.;29:71-110.

51. Buck L, Axel R.1991. A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. Cell;65:175-187.

52. Jackson T.1991. Structure and function of G protein coupled receptors. Pharmac. Ther.;50:425-442.

53. McLaughlin S, McKinnon P, Robichon A, Spickofsky N, Margolskee R.1993. Gustducin and transducin: a tale of two G proteins. Ciba Foundation Symposium;179:186-200.

54. Lambert D.1993. Signal transduction: G protein and second messengers. British Journal of Anaesthesia;71:86-95.

55. Probst WC, Snyder LA, Schuster DI, Brosius J, Sealfon SC.1992. Sequence alignment of the G-protein coupled receptor superfamily. DNA and Cell Biology;11(1):1-20.

56. Birnbaumer L, Abramowitz J, Brown A.1990. Receptor-effector coupling by G proteins. Biochimica et Biophysica Acta;1031:163-224.

57. Clapham DE, Neer EJ.1993. New roles for G-protein beta-gamma dimers in transmembrane siganlling. Nature;365:403-406.

58. Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ.1991. Model systems for the study of seven-transmembrane segment receptors. Annu. Rev. Biochem.;60:653-688.

59. Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG, Lefkowitz RJ.1989. Phosphorylation sites on two domains of the beta 2-adrenergic receptor are involved in distinct pathways of receptor desensitization. Journal of Biological Chemistry;264(21):12657-65.

60. Lefkowitz RJ.1993. G Protein-Coupled Receptor Kinases. Cell;74:409-412.

61. Haribabu B, Snyderman R.1993. Identification of additional members of human G-protein-coupled receptor kinase multigene family. Proc. Natl. Acad. Sci. USA;90:9398-9402.

62. Blumer KJ, Reneke JE, Thorner J.1988. The STE2 gene product is the ligand-binding component of the alpha-factor receptor of Saccharomyces cerevisiae. Journal of Biological Chemistry;263(22):10836-42.

63. Richardson RM, Kim C, BNenvoic JL, Hosey MM.1993. Phosphorylation and desensitization of human m2 muscarinic choliergic receptors by two isoforms of the beta-adrenergic receptor kinase. The Journal of Biological Chemistry;268(18):13650-13656.

64. Wilden U, Kuhn H.1982. Light-dependent phosphorylation of rhodopsin: number of phosphorylation sites. Biochemistry;21:3014-3022.

65. Ishii K, Chen J, Ishii M, et al.1994. Inhibition of thrombin receptor signaling by a G-protein coupled receptor kinase. The Journal of Biological Chemistry;269(2):1125-1130.

66. Leeb LL, Cotecchia S, DeBlasi A, Caron MG, Lefkowitz RJ.1987. Regulation of adrenergic receptor function by phosphorylation. I. Agonist-promoted desensitization and phosphorylation of alpha 1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT1 MF-2 smooth muscle cells. Journal of Biological Chemistry;262(7):3098-105.

67. Ali H, Richardson RM, Tomhave ED, Didsbury JR, Snyderman R.1993. Differences in phosphorylation of formylpeptide and C5a chemoattractant receptors correlate with differences in desensitization. The Journal of Biological Chemistry;268(32):24247-24254.

68. Schleicher S, Boekhoff I, Arriza J, Lefkowitz RJ, Breer H.1993. A beta-adrenergic receptor kinase-like enzyme is involved in olfactory signal termination. Proc. Natl. Acad. Sci. USA;90:1420-1424.

69. Quintana J, Hipkin RW, Sanchez-Yague J, Ascoli M.1994. Follitropin (FSH) and phorbol ester stimulate the phosphorylation of the FSH receptor in intact cells. The Journal of Biological Chemistry;269(12):8772-8779.

70. Kwatra MM, Schwinn DA, Schreurs J, et al.1993. The substance P receptor, which couples to Gq/11, is a substrate of beta-adrenergic receptor kinase 1 and 2. The Journal of Biological Chemistry;268(13):9161-9164.

71. Wilson CJ, Applebury ML.1993. Arresting G-protein coupled receptor activity. Current Biology;3(10):683-686.

72. Lohse M, Benvoic J, Codina J, Caron M, Lefkowitz R.1990. Beta-arrestin: A protein that regulates beta-adrenergic receptor function. Science;248:1547-1550.

73. Sterne-Marr R, Gurevich VV, Goldsmith P, et al.1993. Polypeptide variants of beta-arrestin and arrestin 3. J. Biol Chem.;268:15640-15648.

74. von Zastrow M, Kobilka BK.1992. Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. Journal of Biological Chemistry;267(5):3530-8.

75. Moro O, Lameh J, Sadée W.1993. Serine- and Threonine-rich domain regulates internalization of muscarinic cholinergic receptors. The Journal of Biological Chemistry;268(10):6862-6865.

76. Benya R, Fathi Z, Battey J, Jensen R.1993. Serines and threonines in the gastrin-releasing peptide receptor carboxyl terminus mediate internalization. The Journal of Biological Chemistry;268(27):20285-20290.

77. Barak L, Tiberi M, Freedman N, Kwatra M, Lefkowitz R, Caron M.1994. A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta2-adrenergic receptor sequestration. The Journal of Biological Chemistry;269(4):2790-2795.

78. Goldstein J, Anderson R, Brown M.1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature;279:679-685.

79. Ghinea N, Hai M, Groyer-Picard M-T, Houllier A, Schoëvaërt D, Milgrom E.1992. Pathways of internalization of the hCG/LG receptor: Immunoelectron microscopic studies in Leydig cells and transfected L-cells. The Journal of Cell Biology;118(6):1347-1358.

80. Strosberg A.1991. Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. Eur. J. Biochem.;190:1-10.

81. Wang L, Mantey S, Lin J, Frucht H, Jensen R.1993. Ligand bindign, internalization, degradation and regulation by guanine nucleotides of bombesin receptor subtypes: A comparative study. Biochimica et Biophysica Acta;1175(2):232-242.

82. Anderson K, Murahashi T, Dostal D, Peach M.1993. Morphological and biochemical analysis of angiotensin II internalization in cultured rat aortic smooth muscle cells. American Journal of Physiology;264(1 pt. 1):C179-188.

83. Yamada M, Yamada M, Richelson E.1993. Role of signal transduction systems in neurotensin receptor down-regualtion induced by agonist in murine neuroblastoma clone N1E-115 cells. Journal of Pharmacology and Experimental Therapeutics;267(1):128-133.

84. Hoxie J, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass L.1993. Internalization and recycling of activated thrombin receptors. Journal of Biological Chemistry;268(18):13756-13763.

85. Lutz W, Sanders M, Salisbury J, Lolait S, O'Carroll A, Kumar R.1993. Vasopressin receptor-mediated endocytosis in cells transfected with V1-type vasopressin receptors. Kidney International;43(4):845-852.

86. Maeda S, Lameh J, Mallet WG, Philip M, Ramachandran J, Sadee W.1990. Internalization of the Hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop. Febs Letters;269(2):386-8.

87. Van Epps DE, Simpson SJ, Johnson R.1993. Relationship of C5a receptor modulation to the functional responsiveness of human polymorphonuclear leukocytes to C5a. Journal of Immunology;150(1):246-252.

88. Nussenzveig D, Heinflink M, Gershenorn M.1993. Agonist-stimulated internalization of the thyrotropin-releasing hormone receptor is dependent on two domains in the receptor carboxyl terminus. Journal of Biological Chemistry;268(4):2389-2392.

89. Roberts R, Gullick W.1990. Bradykinin receptors undergo ligand-induced desensitization. Biochemistry;29:1975-1979.

90. Hausdorff W, Campbell P, Ostrowski J, Yu S, Caron M, Lefkowitz R.1991. A small region of the beta-adrenergic receptor is selectively involved in its rapid regulation. Proc. Natl. Acad. Sci. USA;88:2979-2983.

91. Campbell P, Hnatowich M, O'Dowd B, Caron M, Lefkowitz R, Hausdorff W.1990. Mutations fo the human beta2-adrenergic receptor that impair coupling to Gs interfere with receptor down-regulation but not sequestration. Molecular Pharmacology;39(192-198).

92. Valiquette M, Bonin H, Hnatowich M, Caron M, Lefkowitz R, Bouvier M.1990. Involvement of tyrosine residues located in the carboxyl tail of the human beta2-adrenergic receptor in agonist-induced down-regulation of the receptor. Proc. Natl. Acad. Sci. USA;87:5089-5093.

93. Bouvier M, Collins S, O'Dowd B, et al.1989. Two distinct pathways for cAMP-mediated down-regulation of the beta2-adrenergic receptor. The Journal of Biological Chemisty;264(28):16786-16792.

94. Akamizu T, Ikuyama S, Saji M, et al.1990. Cloning, chromosomal assignment, and regulation of the rat thryrotropin receptor: Expression of the gene is regulated by thyrotropin, agents that increase cAMP levels, and thyroid autoantibodies. Proc. Natl. Acad. Sci. USA;87:5677-5681.

95. Wang S-Z, Hu J, Long R, Pou W, Forray C, El-Fakahany E.1990. Agonist-induced down-regulation of m1 muscarinic receptors and reduction of their mRNA levels in a transfected cell line. FEBS Letters;276(1,2):185-188.

96. Izzo N Jr., Seidmen C, Collins S, Colucci W.1990. alpha1-adrenergic receptor mRNA level is regulated by norepinephrine in rabbit aortic smooth muscle cells. Proc. Natl. Acad. Sci. USA;87:6268-6271.

97. Lory P, Richard S, Rassendren F, Tiaho F, Nargeot J.1989. Electrophysiological expression of endothelin and angiotensin receptors in *Xenopus* oocytes injected with rat heart mRNA. FEBS Letters;258(2):289-292.

98. Shimada S, Spivak C, Uhl G.1991. Endothelin receptor: a profoundly desensitizing receptor expressed in Xenopus oocytes. European Journal of Pharmacology;193(1):123-5.

99. Cyr C, Kris R.1993. Desensitization of human endothelin A receptor. Journal of Cardiovascular Pharmacology;22(Suppl. 8):S11-S14.

100. Cyr C, Rudy B, Kris R.1993. Prolonged desensitization of the human endothelin A receptor in Xenopus oocytes. The Journal of Biological Chemistry;268(35):26071-26074.

101. Yue TL, Nambi P, Wu HL, Feuerstein G.1991. Endothelin receptor binding and cellular signal transduction in neurohybrid NG108-15 cells. Neuroscience;44(1):215-22.

102. Cozza E, Vila M, Gomez-Sanchez C.1990. ET1 receptors in C-6 cells: homogous down-regulation and modulation by protein kinase C. Molecular and cellular endocrinology;70:155-164.

103. Lin W-W, Lee C, Chuang D-M.1990. Comparative studies of phosphoinositide hydrolysis induced by endothelin-related peptides in cultured cerebellar astrocytes, C-6-glioma and cerebellar granule cells. Biochemical and Biophysical Research Communications;168(2):512-519.

104. Stojilkovic SS, Balla T, Fukuda S, et al.1992. Endothelin ETA receptors mediate the signaling and secretory actions of endothelins in pituitary gonadotrophs. Endocrinology;130(1):465-74.

105. Gandhi CR, Behal RH, Harvey SA, Nouchi TA, Olson MS.1992. Hepatic effects of endothelin. Biochem J.;287:879-904.

106. Yang C, Yo Y-L, Ong R, Hsieh J-T.1994. Endothelin- and Sarafotoxin-induced phosphoinositide hydrolysis in cultured canine tracheal smooth muscle cells. Journal of Neurochemistry;62(4):1440-1448.

107. van Heugten H, Bezstarosti K, Dekkers DH, Lamers JM.1993. Homologous desensitization of the endothelin-1 receptor mediated phosphoinositide response in cultured neonatal rat cardiomyocytes. Journal of Molecular and Cellular Cardiology;25(1):41-52.

108. Resink T, Scott-Burden T, Boulanger C, Weber E, Buhler F.1990. Internalization of endothelin by cultured human vascular smooth muscle cells: Characterization and physiological significance. Molecular Pharmacology;38:244-252.

109. Thomas CP, Baldi E, Simonson MS, Kester M, Dunn MJ.1991. Endothelin receptors and coupled GTP-binding proteins in glomerular mesangial cells. Journal of Cardiovascular Pharmacology;.

110. Hirata Y, Yoshimi H, Takaichi S, Yanagisawa M, Masaki T.1988. Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. Febs Letters;239(1):13-7.

111. Hollenberg SM, Shelhamer JH, Cunnion RE.1993. Tachyphylaxis to the vasopressor effects of endothelin in rat aortic rings. American Journal of Physiology;.

112. Ihara M, Noguchi K, Saeki T, et al.1992. Biological profiles of highly potent novel endothelin antagonists selective for the ETA receptor. Life Sci.;50:247-255.

113. Ekelund U, Albert U, Edvinsson L, Mellander S.1993. In-vivo effects of endothelin-1 and ETA receptor blockade on arterial, venous and capillary functions in skeletal muscle. Acta Physiol Scand;148:273-283.

114. Ito H, Hirata Y, Hiroe M, et al.1991. Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. Circ. Res.;69:209-215.

115. Ito H, Hiroe M, Hirata Y, et al.1994. Endothelin ETA receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload. Circulation;89(5):2198-2203.

116. Marsault R, Vigne P, Breittmayer J, Frelin C.1991. Kinetics of vasocontrictor action of endothelins. Am. J. Physiol.;261:C986-C993.

117. Kennelly P, Krebs E.1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. of Biol. Chem.;266(24):15555-15558.

118. Resink T, Scott-Burden T, Weber E, Buhler F.1990. Phorbol ester promotes a sustained down-regulation of endothelin receptors and cellular responses to endothelin in human vascular smooth muscle cells. Biochemical and biophysical research communications;166(3):1213-1219.

119. Chomczynski P, Sacchi N.1987. Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. Analytical Biochemistry;162:156-159.

120. Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: A Laboratory Manual.Cold Spring Harbor Laboratory, New York: 1989

121. Graham F, van der EB A.1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology;52:456.

122. McDowell EM&T B.F.1976. Histologic fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab Med.;100:405-414.

123. Bacollao R.1989. Preservation of biological specimens for observation in a confocal fluorescence microscope and operational principles of confocal fluorescence microscopy. Methods in Cell Biology;31:437-452.

124. Muldoon L, Rodland K, Forsythe M, Magun B.1989. Stimulation of phophatidylinositol hydrolysis, diacylglycerol release, and gene expression in response to endothelin, a potent new agonist for fibroblasts and smooth muscle cells. J. Bio. Chem.;264:8529-8536.

125. Chinkers M, Wilson E.1992. Ligand-independent oligomerization of natiuretic peptide receptors. Identification of heteromeric receptors and a dominant negative mutant. Journal of Biologcal Chemistry;267(26):18589-18597.

126. Hagiwara H, Kozuka M, Sakaguchi H, Eguchi S, Ito T, Hirose S.1991. Separation and purification of 34- and 52-kDa species of bovine lung endothelin receptors and identification of the 34-kDa species as a degradation product. Journal of Cardiovascular Pharmacology;.

127. Kozuka M, Ito T, Hirose S, Lodhi KM, Hagiwara H.1991. Purification and characterization of bovine lung endothelin receptor. Journal of Biological Chemistry;266(25):16892-6.

128. Saiki R, Scharf S, Faloona F, et al.1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science;230:1350-1354.

129. Sanger F, Nicklen S, Coulson A.1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA;74(12):5463-5467.

130. Hopp T, Prickett K, Price V, et al.1988. A short polypeptide marker sequence useful for recombinant protein indentification and purification. Biotechnology;6:1204-1210.

131. Sibley DR, Benovic JL, Caron MG, Lefkowitz RJ.1987. Regulation of transmembrane signaling by receptor phosphorylation. Cell;48(6):913-22.

132. Pearson R, Kemp B.1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods in Enzymology;200:62-81.

133. Hirata Y, Yoshimi H, Takata S, et al.1988. Cellular mechanism of aciton by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. Biochemical and Biophysical Research Communications;154(3):868-875.

134. Snutch T.1988. The use of Xenopus oocytes to probe synaptic communications. TINS;11(6):250-256.

135. Stanimirovic D, Yamamoto T, Uematsu S, Spatz M.1994. Endothelin-1 receptor binding and cellular signal transduction in cultured human brain endothelial cells. Journal of Neurochemistry;62:592-601.

136. Simonson M, Herman W.1993. Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signaling by endothelin-1. The Journal of Biological Chemistry;268(13):9347-9357.

137. Williams D Jr., Jones K, Alves K, Chan C, Hollis G, Tung J-S.1993. Characterization of cloned human endothelin receptors. Life Sciences;53:407-414.

138. MacCumber M, Ross C, Snyder S.1990. Endothelin in brain: Receptors, mitogenesis, and biosynthesis in glial cells. Proc. Natl. Acad. Sci. USA;87:2359-2363.

139. Dascal N.1987. The use of Xenopus oocytes for the study of ion channels. CRC Critical Reviews in Biochemistry;22(4).

140. Goldstein J, Brown M, Anderson R, Russel D, Schneider W.1985. Receptor-mediated endcytosis: concepts emerging from LDL receptor system. Annu. Rev. Cell. Biol.;1:1-39.

141. Marsault R, Feolde E, Frelin C.1993. Receptor externalization determines sustained contractile responses to endothelin-1 in the rat aorta. American Journal of Physiology;.

142. Martin ER, Brenner BM, Ballermann BJ.1990. Heterogeneity of cell surface endothelin receptors. Journal of Biological Chemistry;265(23):14044-9.

143. Hashido K, Gamou T, Adachi M, et al.1992. Truncation of N-terminal extracellular or C-terminal intracellular domains of human ETA receptor abrogated the binding activity to ET-1. Biochemical and Biophysical Research Communications;187(3):1241-1248.

144. Kondoh M, Miyazaki H, Watanabe H, et al.1990. Isolation of anti-endothelin receptor monoclonal antibodies for use in receptor characterization. Biochemical and Biophysical Research Communications;172(2):503-510.