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Research Article

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Endothelin Receptor Is Coupled to Phospholipase C via a Pertussis Toxin-insensitive Guanine Nucleotide-binding Regulatory Protein in Vascular Smooth Muscle Cells

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Abstract

The mechanisms of endothelin-1 (ET) actions were investigated in cultured rat aortic vascular smooth muscle A-10 cells. The A-10 cells have a single class of high affinity binding sites for ET with an apparent M_r of 65,000–75,000 on SDS-PAGE. Stimulation of cells with ET induces mobilization of Ca^{2+} from both intra- and extracellular pools to produce a biphasic increase in cytoplasmic free Ca^{2+} concentration. ET increases cellular levels of inositol trisphosphate and 1,2-diacylglycerol, indicating activation of phospholipase C by ET. ET stimulates production of inositol phosphates in membranes prepared from A-10 cells in the presence of guanosine 5′-*O*-(thiotriphosphate) (GTPγS), but not in its absence. Further, specific binding of ^{125}I-labeled ET to A-10 cell membranes is shown to be inhibited by GTPγS in a dose-dependent manner. Treatment of A-10 cells with pertussis toxin induces ADP-ribosylation of a 41,000-D membrane protein but fails to block the ET-induced increases in inositol phosphate production and Ca^{2+} mobilization. These results indicate that the receptor for ET is coupled to phospholipase C via a guanine nucleotide-binding regulatory protein which is distinct from the pertussis toxin substrate in A-10 cells. (*J. Clin. Invest.* 1990. 85:653–658.) phosphoinositide hydrolysis • inositol phosphate • 1,2-diacylglycerol • Ca^{2+} channel • vasoconstriction

Introduction

Endothelin-1 (ET),[1] a novel peptide produced by vascular endothelial cells, is one of the most potent vasoconstrictors known so far (1, 2). Our previous studies demonstrated that activation by ET of the voltage-dependent Ca^{2+} channel plays an important role in ET-induced vasoconstriction (1–3). Subsequent studies by us and other investigators have revealed that ET also stimulates phospholipase C to produce inositol trisphosphate (IP_3) and to mobilize intracellular Ca^{2+} in vascular smooth muscle (4–9) and other types of cells (10, 11). These results suggest that ET activates the dual transmembrane signaling pathways in the target cells, i.e., inositol phospholipid hydrolysis and Ca^{2+} channel gating.

Recent studies have established a pivotal role for the hydrolysis products of inositol phospholipids as second messengers in actions of numerous hormones (12, 13). In many cases receptor stimulation by these Ca^{2+}-mobilizing hormones is coupled to phospholipase C activation via guanine nucleotide-binding regulatory proteins (G proteins) (14, 15). In some instances, however, the activation of phospholipase C is a secondary event resulting from either agonist-induced stimulation of Ca^{2+} influx across the plasma membrane (16, 17) or some other mechanisms including phosphorylation by a receptor-tyrosine kinase of phospholipase C (18). At present, the nature of ET receptor(s) and the mechanism by which the receptor activation is coupled to phospholipase C stimulation are not known.

In the present study, to understand in depth ET actions in vascular smooth muscle, we tried to characterize the receptor and the signal transduction mechanisms of ET using cultured rat aortic vascular smooth muscle cells (A-10 cells). We concluded from the results that ET binds to and activates a single class of high affinity receptors which are coupled to phospholipase C via a pertussis toxin-insensitive G protein.

Methods

Cell culture and materials. A-10 cells were obtained from American Type Culture Collection through Dainippon Seiyaku (Kyoto, Japan). Cells were grown in DME (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FCS (M.A. Bioproducts, Walkersville, MD), 100 μg/ml streptomycin, and 100 U/ml penicillin G (Meiji Seika, Tokyo, Japan) at 37°C in a fully humidified atmosphere of 5% CO_2 in air. For experimental purposes, confluent cells were serum-deprived by culturing in DME containing 0.3% BSA for 24 h.

Human ET, obtained from Peptide Institute (Osaka, Japan), was dissolved at 10^{-4} M in Dulbecco's PBS containing 0.5% BSA and stored in aliquots at −20°C. Dilutions were made immediately before use. Fura-2 acetoxymethyl ester was purchased from Wako (Tokyo, Japan). Nicardipine was generously provided by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). *Myo*-[2-^{3}H]Inositol (14.3 Ci/mmol), adenosine 5′-[γ-^{32}P]triphosphate (3,000 Ci/mmol), and [adenylate-^{32}P]nicotinamide adenine dinucleotide ([^{32}P]NAD) were purchased from Dupont/New England Nuclear Research Products (Boston, MA). ^{125}I-ET (~ 2,000 Ci/mmol) was kindly provided by Dr. S. Kimura. Pertussis toxin was purchased from Kaken Seiyaku (Kyoto,

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ET, endothelin-1; GTPγS, guanosine 5′-*O*-(thiotriphosphate); IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; [^{32}P]NAD, [adenylate-^{32}P]nicotinamide; PCA, perchloric acid.

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Japan). Silica gel 60 TLC plate was purchased from E. Merck (Darmstadt, FRG).

^{125}I-ET binding experiments. A-10 cells in a 24-well plate were washed with medium A (140 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$ 11 mM glucose, 5 mM Hepes [pH 7.4], and 0.2% BSA) and incubated in medium A containing various concentrations of ^{125}I-labeled ET for 30 min at 25°C. After washing cells three times with ice-cold medium A, the cell-bound radioactivity was counted. Specific binding was determined as total binding minus nonspecific binding in the presence of 10^{-7} M unlabeled ET.

For experiments to examine the effect of a guanine nucleotide on ^{125}I-ET binding to A-10 cell membranes, cells in a 100-mm dish were washed twice with PBS, scraped into a solution containing 250 mM sucrose, 1 mM $MgCl_2$, and 10 mM Tris/HCl (pH 7.4), and homogenized with a glass–glass homogenizer at 4°C. After centrifugation at 200 *g* for 10 min at 4°C the supernatant was centrifuged at 10,000 *g* for 5 min at 4°C and the resulting pellet was resuspended in a 10-mM Tris/HCl buffer (pH 7.4) containing 120 mM NaCl and 3 mM $MgCl_2$. ^{125}I-ET was incubated with 25 μg membrane protein in the presence of various concentrations of guanosine 5′-*O*-(thiotriphosphate) (GTPγS) for 24 h at 4°C. The incubation mixtures were filtered through GF/F filters (Whatman Laboratory Products Inc., Clifton, NJ) and the radioactivity associated with the filters was measured with a gamma counter. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled ET.

Affinity cross-linking of ^{125}I-ET. A-10 cells in a 35-mm plastic dish were incubated at 25°C in medium A containing 3×10^{-10} M ^{125}I-ET in the presence or absence of 10^{-7} M unlabeled ET for 30 min. The cells were washed two times with PBS and incubated with 1 mM ethylene glycolbis (succinimidyl succinate) in BSA-free medium A at 22°C for 50 min. The cells were rapidly washed twice with PBS and solubilized in 0.5 ml of the sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2.3% SDS, and 5% β-mercaptoethanol). After boiling at 100°C for 3 min the samples were analyzed by 10% SDS-PAGE followed by autoradiography.

Determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). A-10 cells were dispersed by 0.025% trypsin treatment and loaded with fura-2 by incubating cells in medium A containing 4 μM fura-2 acetoxymethyl ester for 45 min at 20°C. The fluorescence of fura-2-loaded cells was measured with a CAF-100 spectrofluorimeter (Japan Spectroscopy, Inc., Tokyo, Japan) with excitation at 340 and 380 nm and emission at 500 nm as previously described (11). The free Ca^{2+} concentration was calculated from the measurements of the ratio of fluorescence intensities as described by Grynkiewicz et al. (19).

Measurements of inositol phosphate production and cellular 1,2-diacylglycerol content. For measurement of inositol phosphate production, cells were prelabeled with *myo*-[2-^{3}H]inositol (4 μCi/ml) in DME containing 0.3% BSA for 24 h and stimulated by various concentrations of ET for indicated time periods at 37°C. The reaction was stopped by replacing media with ice-cold 10% perchloric acid (PCA) solution. The neutralized PCA extract was analyzed for inositol phosphates using Dowex columns as described (20). The cellular content of 1,2-diacylglycerol was measured by an enzymatic method using an *Escherichia coli* diacylglycerol kinase according to the method described by Preiss et al. (21).

Measurement of inositol phosphate production in the plasma membranes. A-10 cell membranes were obtained as described above, except that the homogenization buffer contained 250 mM sucrose, 1 mM EGTA, 1 mM ATP, 0.1 mM $MgCl_2$, and 10 mM Tris/HCl (pH 7.4), and membranes were resuspended in a buffer containing 5 mM $MgCl_2$, 1 mM EGTA, 10 mM LiCl, and 50 mM Hepes/NaOH (pH 7.4). The membranes were incubated with 10^{-7} M ET and/or various concentrations of GTPγS in the presence of 10^{-7} M free Ca^{2+} concentration determined by a Ca^{2+}-EGTA buffer (22) at 37°C for 1 min. The reaction was terminated by addition of PCA (a final concentration of 10%) and analyzed for inositol phosphates as described above.

In vitro ADP-ribosylation of A-10 cell membranes. A-10 cells, either untreated or treated with 10 ng/ml pertussis toxin for 24 h, were washed twice with PBS, scraped, and homogenized in a homogenization buffer containing 5 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris/HCl (pH 7.4). The homogenates were briefly sonicated and centrifuged at 10,000 *g* for 5 min at 4°C. The resulting pellet was resuspended in a homogenization buffer. The membranes (30 μg) were incubated with 25 μg/ml pertussis toxin for 75 min at 25°C in 20 mM Tris/HCl buffer (pH 7.5) containing 10 mM thymidine, 40 μM [^{32}P]-NAD, 1 mM ATP, 0.5 mM EDTA, 20 mM dithiothreitol, and 1 mM $MgCl_2$. The reaction was terminated with TCA (a final concentration of 10%) and the pellet was solubilized in the SDS-sample buffer and analyzed by 8% SDS-PAGE followed by autoradiography.

Results

Specific binding and affinity cross-linking of ^{125}I-ET to A-10 cells. To characterize the specific binding site for ET in A-10 cells, a radioligand binding assay was performed using [^{125}I]ET (Fig. 1). Specific binding was > 76% of total binding at any concentration of [^{125}I]ET tested and was a saturable process (Fig. 1 *A*). Scatchard analysis of the data revealed a single class of high affinity binding sites with a K_d of 3×10^{-10} M and a B_{max} of 67,000 binding sites per cell (Fig. 1 *B*). Affinity cross-linking of [^{125}I]ET to receptors with a cross-linking reagent, ethylene glycolbis (succinimidyl succinate), revealed the presence of a single major band with an M_r of 65,000–75,000 on the SDS-PAGE (Fig. 1 *C*). In the presence of 300-fold excess unlabeled ET this band was completely abolished.

Effect of ET on $[Ca^{2+}]_i$ in A-10 cells. The ET-induced Ca^{2+} mobilization was studied by measurement of changes in $[Ca^{2+}]_i$, using fura-2 as a Ca^{2+} indicator (Fig. 2). The baseline

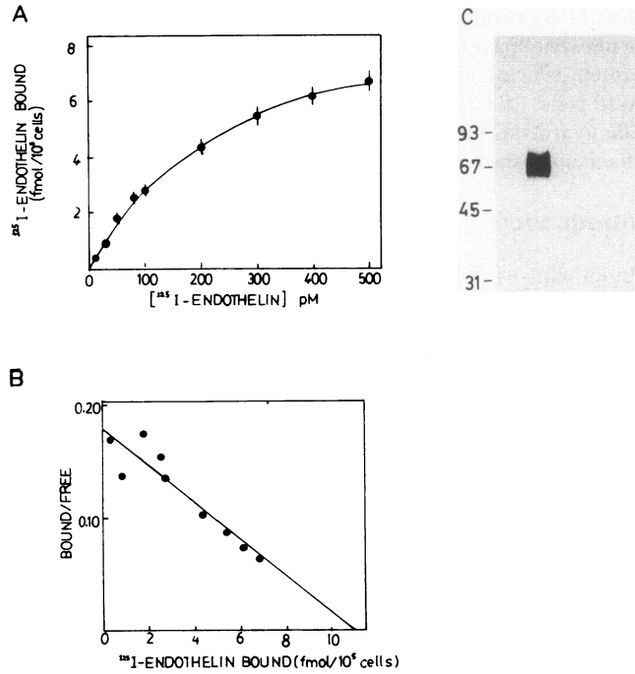

Figure 1. Binding of ^{125}I-ET to intact A-10 cells. *A*, Specific binding of ^{125}I-ET. The data represent the means±SD of three determinations. *B*, Scatchard plot for specific binding of ^{125}I-ET. *C*, Affinity cross-linking of ^{125}I-ET in the absence (*left*) or presence (*right*) of an excess amount of nonlabeled ET. Molecular weight standards ($\times 10^{-3}$) are shown on the left. The gel was exposed to a film for 12 d at −80°C.

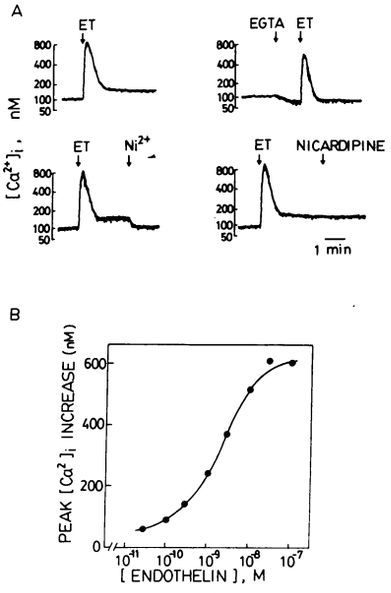

Figure 2. Effect of ET on $[Ca^{2+}]_i$. *A*, Effects of EGTA (2×10^{-3} M), Ni^{2+} (1×10^{-3} M), and nicardipine (1×10^{-6} M) on 10^{-7} M ET-induced increase in $[Ca^{2+}]_i$. Test substances were applied at arrows and were present throughout each recording. Tracings are representative of at least four experiments. *B*, Dose-response curve of ET-induced increases in $[Ca^{2+}]_i$. Each value expresses the mean of four to six determinations.

$[Ca^{2+}]_i$ was 92±11 nM (mean±SD, n = 34). Addition of 10^{-7} M ET induced a prompt rise in $[Ca^{2+}]_i$, which peaked within 15 s of ET addition with a peak value of 686±24 nM (n = 4) (Fig. 2 *A*). The $[Ca^{2+}]_i$ then fell to a lower plateau level (160±4 nM at 3 min, n = 4), which was significantly higher than the baseline value. The peak $[Ca^{2+}]_i$ increase was dose dependent and became greater with increasing doses of ET (Fig. 2 *B*). The response was saturated with 3×10^{-8} M ET. The ED_{50} for ET-induced increases in $[Ca^{2+}]_i$ was estimated to be $\sim 3 \times 10^{-9}$ M. When extracellular Ca^{2+} was lowered by adding 2 mM EGTA (free Ca^{2+}, 200 nM), the basal $[Ca^{2+}]_i$ declined to a new steady-state level (Fig. 2 *A*). Under such a condition, ET (10^{-7} M) still induces the initial Ca^{2+} transient, but not the second plateau of the $[Ca^{2+}]_i$ response. In the presence of 1.25 mM $CaCl_2$, addition of 1 mM Ni^{2+}, an inorganic Ca^{2+} channel blocker, promptly abolished the second plateau phase of the $[Ca^{2+}]_i$ response to ET (Fig. 2 *A*). These findings suggest that ET induces Ca^{2+} mobilization from both intra- and extracellular pools. Previous studies suggested that ET acts on porcine coronary artery smooth muscle cells to activate a dihydropyridine-sensitive Ca^{2+} channel. However, in the present study a dihydropyridine Ca^{2+} channel antagonist, nicardipine, did not inhibit the plateau phase of the $[Ca^{2+}]_i$ response to ET at concentrations up to 1×10^{-6} M in A-10 cells (Fig. 2 *A*).

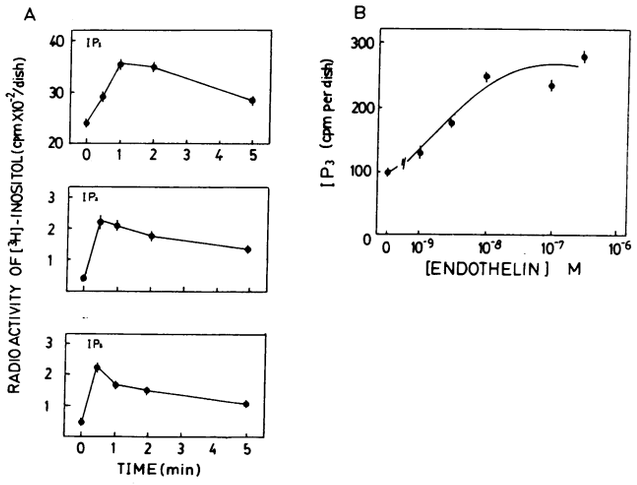

Figure 3. Effect of ET on inositol phosphate generation. *A*, Time course of changes in inositol phosphates when [^{3}H]inositol-labeled A-10 cells were stimulated with 10^{-7} M ET. *B*, Dose-response curve of ET-induced increases in IP_3. The data represent the mean±SD of three determinations.

Effects of ET on inositol phosphate and 1,2-diacylglycerol production in A-10 cells. As shown in Fig. 3 *A*, addition of 10^{-7} M ET induced rapid rises in inositol bisphosphate (IP_2) and IP_3. The levels of IP_2 and IP_3 peaked at 30 s and then gradually declined for the next 4 min. Inositol monophosphate (IP_1) rose less rapidly than IP_2 and IP_3, reached a peak at 1 min, and then declined. As shown in Fig. 3 *B*, ET-induced production of IP_3 increased dose dependently. The response was saturated at 10^{-7} M ET. The ED_{50} value for the response was estimated to be $\sim 3 \times 10^{-9}$ M. The ET-evoked inositol phosphate productions were not significantly affected by extracellular Ca^{2+} removal, suggesting that ET-induced stimulation of inositol phosphate production is not a result of stimulation of Ca^{2+} influx across the plasma membrane (data not shown).

Table I. Effect of ET on Cellular 1,2-Diacylglycerol Content in A-10 Cells

Time	1,2-Diacylglycerol
	nmol/dish
0	0.193±0.007
20 s	0.265±0.006
1 min	0.313±0.016
2 min	0.367±0.013
5 min	0.437±0.018

Confluent monolayer cells were incubated with 10^{-7} M ET for indicated time periods and 1,2-diacylglycerol content was measured. Data are the mean±SD of three determinations.

Table I shows the time course of ET-induced changes in cellular 1,2-diacylglycerol content. Within 20 s of ET (10^{-7} M) addition, cellular 1,2-diacylglycerol content increased significantly and continued to rise for 5 min, when the maximal value of 2.3-fold over the basal level was observed. These results indicate that ET activates phospholipase C, which catalyzes the breakdown of polyphosphoinositides in A-10 cells.

Effects of GTPγS on ET binding and ET-induced inositol phosphate production in A-10 cell membranes. To examine whether the receptor for ET was coupled to a G protein, the effect of a nonhydrolyzable GTP analogue, GTPγS, on the

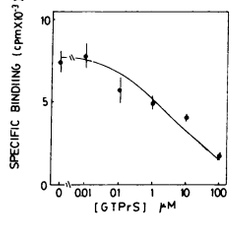

Figure 4. Effect of GTPγS on the specific binding of ^{125}I-ET to A-10 membranes. A-10 membranes were incubated with ^{125}I-ET in the presence of various concentrations of GTPγS for 24 h at 4°C and specific binding of ^{125}I-ET to membranes was determined. Values are the mean±SD of three determinations.

Table II. Dependence of ET-stimulated Inositol Phosphate Productions on GTPγS

Additions	IP_1	IP_2	IP_3
		cpm	
None	1,124±100	52±10	12±2
ET	1,228±71	72±8	15±5
GTPγS	1,166±90	93±11	16±7
GTPγS + ET	1,921±172	319±30	34±2

[^{3}H]Inositol-prelabeled plasma membranes were incubated with 10^{-7} M ET in the presence or absence of 10^{-6} M GTPγS for 1 min at 37°C. Free Ca^{2+} concentration in the assay buffer was 10^{-7} M. Results are mean±SD of three determinations.

Table III. Effect of Pertussis Toxin Pretreatment on ET-induced Inositol Phosphate Productions in A-10 Cells

	Control		PT-pretreated	
	None	Endothelin	None	Endothelin
		cpm		
IP_1	5,467±617 (100%)	7,085±766 (130%)	5,025±551 (100%)	6,668±498 (133%)
IP_2	78±12 (100%)	178±23 (228%)	64±8 (100%)	177±20 (277%)
IP_3	68±7 (100%)	171±7 (251%)	51±5 (100%)	138±10 (271%)

Confluent A-10 cells were incubated with 10 ng/ml pertussis toxin (*PT*) or vehicle for 24 h and stimulated with 10^{-7} M ET for 1 min. Results are mean±SD of three determinations.

specific binding of ET to membranes prepared from A-10 cells was studied. As shown in Fig. 4, GTPγS inhibited the specific binding of ^{125}I-ET to A-10 membranes in a dose-dependent manner. At 100 μM, GTPγS inhibited the ^{125}I-ET binding by 70%.

We next examined the effect of GTPγS on ET-induced inositol phosphate production in A-10 cell membranes. In the absence of GTPγS, 10^{-7} M of ET alone did not significantly stimulate inositol phosphate production (Table II). GTPγS (10^{-6} M) by itself had a small stimulating effect on inositol phosphate production (80% increase in IP_2 and no increase in IP_1 and IP_3). However, if ET was applied together with GTPγS, a marked increase (510%) in IP_2 production and significant increases in IP_1 and IP_3 (70% in IP_1 and 180% in IP_3) were induced. Fig. 5 shows the dose-dependent effects of GTPγS on ET-induced inositol phosphate (IP_2 plus IP_3) production in A-10 membranes. As little as 10^{-7} M GTPγS significantly enhanced ET-induced inositol phosphate production.

The effect of pertussis toxin pretreatment on ET-induced inositol phosphate production and Ca^{2+} mobilization in A-10 cells. As shown in Table III, exposure of intact A-10 cells to 10 ng/ml of pertussis toxin for 24 h did not significantly affect the following ET-induced inositol phosphate production. The extent of labeling of cellular inositol phospholipids with [^{3}H]-myoinositol did not significantly differ between pertussis toxin–treated and nontreated cells (34,100±10,900 vs. 36,600±3,300 cpm, mean±SD [n = 6]). In cells pretreated with pertussis toxin the peak $[Ca^{2+}]_i$ increment induced by 10^{-7} M ET was smaller by only 20% than in nontreated cells (486±54 vs. 600±95 nM; mean±SD [n = 4]), and the second plateau of the $[Ca^{2+}]_i$ response was comparable between pertussis toxin–treated and nontreated cells (63±4 vs. 62±14 nM). The effectiveness of pertussis toxin pretreatment in intact A-10 cells was proven by the fact shown in Fig. 6 that a 41,000-D protein of the plasma membranes prepared from A-10 cells pretreated with pertussis toxin for 24 h was not significantly ADP-ribosylated in vitro by pertussis toxin in contrast to the membranes prepared from nontreated A-10 cells. These results suggest that a G protein, distinct from the pertussis toxin substrates, coupled the ET receptor to phospholipase C in A-10 cells.

Discussion

Previous studies by us and other investigators demonstrated that ET activates phospholipase C to produce IP_3 and 1,2-diacylglycerol in vascular smooth muscle and other cell types (5–11). The results in the present study confirm these observations and further demonstrate that ET stimulates phospholipase C by a guanine nucleotide-dependent mechanism in cultured vascular smooth muscle A-10 cells. A-10 cells possess a single class of high affinity receptors for ET (Fig. 1). The binding of ET to the receptors on A-10 cell membranes is inhibited by a nonhydrolyzable GTP analogue, GTPγS, in a dose-dependent manner (Fig. 4). The inhibitory modulation by guanine nucleotides of the binding of various agonists to their respective receptors is a well-known characteristic found in the receptors that interact with G proteins (23, 24). Moreover, ET

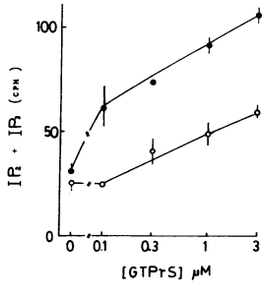

Figure 5. Effect of various concentrations of GTPγS on ET-induced production of inositol phosphate in A-10 membranes. A-10 membranes were incubated with (●) or without (○) 10^{-7} M ET in the presence of various concentrations of GTPγS. The IP_2 and IP_3 produced were measured. Values are the mean±SD of three determinations.

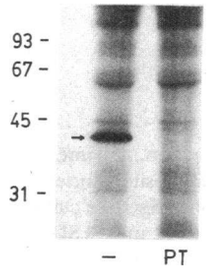

Figure 6. In vitro ADP-ribosylation of a 41-kD protein in plasma membranes prepared from A-10 cells nonpretreated or pretreated with pertussis toxin. A-10 cells were incubated with or without 10 ng/ml of pertussis toxin (*PT*) for 24 h and crude membrane fractions were prepared. The membranes were then incubated with [^{32}P]NAD and 25 μg/ml pertussis toxin. Proteins were resolved by SDS-PAGE followed by autoradiography. Arrow indicates the 41-kD protein. Molecular weight standards ($\times 10^{-3}$) are shown on the left.

stimulates polyphosphoinositide hydrolysis in A-10 cell membranes in a manner strictly dependent on GTPγS (Table II and Fig. 5). These results strongly suggest that the ET receptor is coupled to phospholipase C by a guanine nucleotide-binding regulatory protein (G protein) in A-10 cells. Pretreatment of A-10 cells with pertussis toxin does not affect ET-induced inositol phosphate production (Table III), even though pertussis toxin fully ADP-ribosylates a 41-kD membrane substrate protein under the identical condition (Fig. 6). These findings suggest that a G protein distinct from pertussis toxin–sensitive G proteins, Gi or Go (25), couples the ET receptor to phospholipase C in A-10 cells.

Recent studies on the structure of the G protein–linked receptors, including rhodopsin, adrenergic, muscarinic, and serotoninergic receptors, have revealed that these receptors have considerable similarities in amino acid sequences and share a characteristic membrane-spanning structure (26–28). The molecular weights of these receptors were reported to be in the range of 55,000–80,000 (26–28). In the present study, the affinity cross-linking experiment identified the ET receptor as a single band with an apparent molecular weight of 65,000–75,000 on SDS-PAGE (Fig. 1). These data further support the notion that the ET receptor belongs to the G protein–linked rhodopsin type of receptor superfamily.

The measurements of $[Ca^{2+}]_i$ with fura-2 demonstrate a biphasic pattern of ET-induced Ca^{2+} mobilization: an initial transient increase in $[Ca^{2+}]_i$ due to intracellular mobilization, most likely mediated by inositol 1,4,5-trisphosphate (13), and a second sustained plateau phase (Fig. 2). The second plateau phase of the $[Ca^{2+}]_i$ response to ET is dependent on the presence of extracellular Ca^{2+}, and is blocked by an inorganic Ca^{2+} channel blocker, Ni^{2+}, suggesting that the second plateau phase is mainly due to Ca^{2+} influx across the plasma membrane. Our previous study (3) using a patch clamp technique demonstrated that ET acts on porcine coronary artery smooth muscle to activate the dihydropyridine-sensitive (L-type), voltage-dependent Ca^{2+} channel. However, in A-10 cells a dihydropyridine Ca^{2+} channel antagonist nicardipine does not inhibit the second plateau of the $[Ca^{2+}]_i$ response to ET (Fig. 2). Similar results on cultured vascular smooth muscle cells were recently reported by Mitsuhashi et al. (29). We have found that this is also the case with nonsmooth muscle cells, Swiss 3T3 fibroblasts (11), and MC3T3-El osteoblastic cells (30), which also show a biphasic increase in $[Ca^{2+}]_i$ in response to ET. It is possible that the apparent discrepancy in the nicardipine effects observed with two different methodologies, measurements of $[Ca^{2+}]_i$ with fura-2 and a patch clamp technique, is due to the actual difference in the properties of the Ca^{2+} channel through which Ca^{2+} enters into cells in response to ET stimulation in porcine coronary artery smooth muscle and cultured vascular smooth muscle cells. Comparable studies using both methodologies in each cell type may elucidate this point. Although the mechanism of the Ca^{2+} channel activation by ET in A-10 cells is unknown at present, stimulation of Ca^{2+} influx which is insensitive to dihydropyridine Ca^{2+} channel antagonists is commonly observed with numerous Ca^{2+}-mobilizing hormones in various types of cells (31). The failure of pertussis toxin pretreatment to inhibit the second plateau of the $[Ca^{2+}]_i$ response to ET in A-10 cells suggests that activation of the Ca^{2+} channel by ET is not mediated by the pertussis toxin–sensitive G proteins in these cells as suggested in other types of cells (32). Recent cell-attached patch clamp studies on freshly dispersed porcine coronary artery smooth muscle cells suggested that the activation by ET of the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel is mediated by a readily diffusible second messenger molecule (33). Thus, the Ca^{2+} channel activation might be secondary to the proximal membrane signal transduction events, including the activation of phospholipase C in coronary artery smooth muscle. It would be very interesting to know the precise mechanisms by which ET induces activation of Ca^{2+} channels in coronary artery smooth muscle and other types of cells.

In conclusion, the present results indicate that the receptor activation by ET is coupled to phosphoinositide hydrolysis through a pertussis toxin–insensitive G protein and gating of dihydropyridine-insensitive Ca^{2+} channels, leading to Ca^{2+} mobilization from both intra- and extracellular pools and activation of protein kinase C in cultured aortic vascular smooth muscle. It is suggested that these transmembrane signaling pathways play an important role in mechanisms for ET-induced vasoconstriction (34–36).

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