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Endothelin-1-Induced Mitogenic Responses of Chinese Hamster Ovary Cells Expressing Human Endothelin$_A$: The Role of a Wortmannin-Sensitive Signaling Pathway

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SUMMARY

In the current study, endothelin-1 (ET-1) worked as a mitogen on Chinese hamster ovary cells stably expressing human endothelin$_A$; when applied to serum-deprived cells, ET-1 caused dose-dependent increases in [^{3}H]thymidine incorporation and cell proliferation. No synergism was observed between the effect of ET-1 and that of insulin-like growth factor-1/basic fibroblast growth factor. Both the inhibition of intracellular Ca^{2+} response by phospholipase C inhibitor U73122 and the down-regulation of protein kinase C (PKC) by pretreatment with phorbol 12-myristate-13-acetate (PMA) partially blocked the ET-1-induced mitogenic responses. Wortmannin, a phosphatidylinositol-3-kinase inhibitor, caused dose-dependent inhibition of the ET-1-induced mitogenic responses in both PMA-treated and -untreated cells. Wortmannin also inhibited ET-1-induced increase in phosphatidylinositol trisphosphate formation and activation of mitogen-activated protein kinase (MAPK), whereas it failed to inhibit PMA-induced activation of MAPK. In accordance with its effect on MAPK activation, wortmannin inhibited ET-1-induced activation of Raf-B, whereas it failed to inhibit the effect of PMA. These results suggested the role of a Ca^{2+}/PKC-independent, wortmannin-sensitive signaling pathway that linked ET_A and MAPK cascade in the mitogenic signaling activated by ET_A.

The ETs are a family of vasoactive peptides that includes ET-1, -2, and -3 (1, 2). Since their discovery, numerous studies have described their multiple biological activities, and the peptides have been implicated in a wide variety of physiological functions associated with the cardiovascular, endocrinal, pulmonary, renal, and nervous systems (3, 4). Of the diverse actions of the peptides, mitogenic activities of ETs have attracted much attention because they suggested a possible role of ETs in the pathogenesis of certain clinical conditions. ET-1 has been reported to stimulate DNA synthesis and proliferation of a variety of cells such as SMCs, glomerular mesangial cells, osteoblasts, fibroblasts, and melanocytes (3, 4). Clinical studies have reported elevated tissue and plasma levels of immunoreactive ETs in patients with conditions such as hyperlipoproteinemia (5) or atherosclerosis (6), suggesting the significance of their mitogenic activity on SMCs in atherogenesis. ET-1 has also been identified as an autocrine/paracrine growth factor for some human cancer cell lines (7), suggesting the role of the peptide in oncogenesis or tumor growth. Furthermore, the recent development of transgenic mice with disrupted ET-1 (8) or ET-3 (9) genes revealed the role of these peptides as a growth and/or differentiation factor for definite cell lines in embryogenesis. Despite this accumulating evidence of the physiological/pathological significance of ETs as a mitogen, the receptor subtype and the intracellular signaling pathways responsible for the mitogenic activity remain unclear.

cDNA cloning of cell surface receptors for ETs has identified two subtypes of ETRs, designated ET_A and ET_B, both of

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ABBREVIATIONS: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; CHO, Chinese hamster ovary; ET, endothelin; ETR, endothelin receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PI(3)K, phosphatidylinositol-3-kinase; PIP, phosphatidylinositol monophosphate; PIP_3, phosphatidylinositol trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate 13-acetate; SDS, sodium dodecyl sulfate; SMC, smooth muscle cell; TLC, thin layer chromatography; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IGF-1, insulin-like growth factor-1; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; FCS, fetal calf serum; PI(4)P, phosphatidylinositol-4-monophosphate; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; TCA, trichloroacetic acid; IP, inositol phosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

which belong to a family of G protein-coupled receptors (10, 11). The two subtypes have distinct ligand selectivity; ET_A preferentially binds ET-1 and ET-2, whereas ET_B binds all three isopeptides with similar affinities. Interaction of ET/ETR evokes a complex network of signal transduction involving such effector molecules as PLC, PKC, phospholipase A_2, phospholipase D, receptor-operated and voltage-gated Ca^{2+} channels, Na^+/H^+ antiport, MAPK, and protein tyrosine kinases such as src (3, 4, 12). We therefore addressed which subtype of the ETRs and which effector molecule or molecules are essential for the mitogenic signaling of ETs and whether any other effector molecules are involved.

The cell types mentioned (i.e., SMCs, mesangial cells, osteoblasts, fibroblasts, and melanocytes) predominantly express ET_A, and some studies have demonstrated the ability of an ET_A-specific antagonist (BQ123) to block the mitogenic activity of ET-1 (12–14), suggesting that ET/ET_A interaction is necessary for the mitogenic action of ETs. These studies, however, never excluded the possible role of ET_B, which has been proved to be expressed, on SMCs at least (14–16). A recent finding that the ratio of the densities of ET_A/ET_B expressed on cultured SMCs could change during cell passages[2] is a further complication.

Instead of using subtype-specific agonists and antagonists, as an alternative approach to the study of subtype-specific mitogenic signaling of ET, we generated stable transfectants expressing human ET_A. Three cell lines were chosen: CHOK1 and Ltk^- cells because of the absence of endogenous ETR, and JH4 cells because of the absence of endogenous ET_A and the presence of a low density of ET_B, which does not induce an increase in $[Ca^{2+}]_i$.[3] Of the transfectants obtained from these different cell lines, we found that only the clones derived from CHOK1 cells (CHO/ET_A) exhibited late mitogenic responses ([^{3}H]thymidine incorporation and cell proliferation) when incubated in serum-free medium containing ET-1. The current study was therefore undertaken to elucidate the signaling pathway or pathways that are essential for the mitogenic effect of ET-1 on CHO/ET_A cells.

Experimental Procedures

Materials. Synthetic human ET-1 was obtained from Peptide Institute (Osaka, Japan). BQ123 was obtained from Peninsula Laboratories (Belmont, CA). Human recombinant IGF-1, bFGF, EGF, and platelet-derived growth factor-BB homodimer were purchased from Genzyme (Cambridge, MA). HAM's F-12, phosphate-free RPMI1640, FCS, lipofectamine, and a rabbit polyclonal anti-PKCα antibody were purchased from Life Technologies (Gaithersburg, MD). [^{125}I]ET-1 (74 TBq/mmol), [*methyl*-^{3}H]thymidine (3.1 TBq/mmol), *myo*-[^{3}H]inositol (370 GBq/mmol), carrier-free ^{32}Pi, [γ-^{32}P]ATP (220 TBq/mmol), and MAPK assay kits were obtained from Amersham International (Buckinghamshire, UK). Fura-2/acetoxymethyl ester was obtained from Dojin Chemicals (Tokyo, Japan). U73122 (1-(6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl)-1*H*-pyrrole-2,5-dione) and its structural analogue U73343 (1-(6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl)-2,5-pyrrolidine-dione) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Wortmannin, MBP, PMA, PI, and PI(4)P were obtained from Sigma Chemical Co. (St. Louis, MO). Wortmannin was dissolved in dimethylsulfoxide at 10 mM, stored at −20° in the dark, and diluted with an appropriate buffer just before use. A rabbit polyclonal antibody against Raf-B (C-19), human recombinant MEK-1, and MAPK (p42) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The BCA microprotein assay kit was obtained from Pierce (Rockford, IL), and the cell proliferation assay kit with MTT was obtained from Promega (Madison, WI). Dr. Tadaomi Takenawa (Institute of Medical Science, University of Tokyo) kindly provided us with $PI(4,5)P_2$ purified from bovine brain. All other reagents used were of the purest grade available.

Stable expression of human ET_A. To obtain cell lines stably expressing ET_A, we used SRα promoter-based mammalian expression vector pME18Sf, which carried cDNA construct encoding wild-type human ET_A. Procedures for construction and subcloning of the plasmid have been described previously (17). The plasmid was co-transfected with pSV*neo*^{r} plasmid into the cells by lipofection, with the use of lipofectamine (GIBCO-BRL) according to the manufacturer's instructions. Cell populations expressing *neo*^{r} gene product were selected in F-12/10% FCS containing G418 (0.5 mg/ml). From these selected cell populations, cell clones were isolated by colony lifting and maintained in the same selection medium. The density of receptors expressed in each clone was determined by saturation isotherms of [^{125}I]ET-1 binding to the crude membrane preparations as described previously (18).

[^{3}H]Thymidine incorporation. Cells were cultured onto 96-well plates to ~50% confluence. They were deprived of serum for 24 hr in serum-free F-12, washed with PBS, and then further incubated for 24 hr in serum-free F-12 with ET-1 or other growth factors at the concentrations indicated. The cells were exposed to [^{3}H]thymidine (1 μCi/ml) during the last 4 hr of the incubation, washed twice with ice-cold PBS, and then incubated with 10% (w/v) TCA at 4° for 30 min. After removal of the TCA-soluble material with ethanol, the radioactivity incorporated into the TCA-insoluble fraction was recovered in 0.5 N NaOH and counted with a liquid scintillation counter.

Cell proliferation assay. Cells at ~50% confluence plated onto 96-well plates were fasted for 24 hr, washed with PBS, and then incubated for 48 hr in serum-free F-12 with or without ET-1 at the concentrations indicated. The number of viable cells in each well was estimated by measurement of the rate of mitochondrial metabolism of MTT using a cell proliferation assay kit (Promega). In brief, the cells were exposed to MTT (1 mg/ml) during the last 4 hr of the incubation and then lysed with acidic lysis buffer containing SDS. The absorbances of the lysate at 562 and 630 nm were measured with an EL340 microtiter plate reader (BIO-TEK Instruments, Winooski, VT). Control experiments showed the linear relationship between the absorbance and the cell number up to the cell density of 20,000/well (~0.4 of A_{562nm}–A_{630nm}).

Cell cycle analysis. Cells were plated onto 100-mm dishes and grown to ~50% confluence. They were subjected to serum-deprivation, followed by stimulation with ET-1 or 10% FCS as described above. At the given time points, the cells were washed with PBS and then harvested by trypsinization. They were fixed with 70% ethanol, washed, and then incubated in PBS containing 50 μg/ml propidium iodide and 1 mg/ml RNase A. The DNA content per nucleus was analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson Co., Mansfield, MA).

Measurement of $[Ca^{2+}]_i$. Cells in 100-mm dishes were dispersed by incubation in PBS containing 1 mM EGTA and loaded with Fura-2 by incubation at 20° for 35 min in HEPES-buffered saline (20 mM HEPES, pH 7.4, 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, and 0.1% bovine serum albumin) containing 4 μM Fura-2/acetoxymethyl ester. The Fura-2-loaded cells were washed twice, resuspended in HEPES-buffered saline, and kept at 20°. The fluorescence of the cells was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy Inc., Tokyo, Japan) with excitations at 340 and 380 nm and emission at 500 nm. The $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities as described previously (19).

PI breakdown assay. Cells at ~50% confluence in 24-well plates were incubated for 24 hr in serum-free F-12 containing [^{3}H]inositol

[2] Y. Hirata, personal communication.
[3] H. Ninomiya, unpublished observations.

(5 μCi/ml). The cells were washed once with Krebs-Hensleit buffer with LiCl (110 mM NaCl, 4.5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.7 mM glucose, and 10 mM LiCl) equilibrated with 5% CO_2 and then incubated in 240 μl of the same buffer. After the addition of U73122 or U73343 (30 μl of solution to give a final concentration of 10 μM) or buffer for controls, ET-1 (30 μl of solution) was added at increasing concentrations, and the plates were kept in the CO_2 incubator for 30 min. The incubations were terminated by the addition of 100 μl/well ice-cold 10% perchloric acid and neutralized with 150 mM KOH, and the cellular debris was pelleted by centrifugation. The supernatants were applied to small columns containing Dowex anion-exchange resin AG1X8 (100–200 mesh in the chloride form; Bio-Rad, Hercules, CA) to separate total IPs from inositol. After the column was washed with water, [^{3}H]IPs were eluted with 1 N HCl and counted for radioactivity with a liquid scintillation counter.

PMA treatment of cells and PKC immunoblotting. Cells at ~50% confluence in six-well plates were incubated for 24 hr in serum-free F-12 with or without 100 ng/ml PMA, washed with PBS, and then harvested in 0.25 ml of lysis buffer (10 mM Tris·HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM AEBSF, and 10 μg/ml aprotinin). After brief sonication and centrifugation, the lysate (0.2 ml) was incubated at 4° for 16 hr with 5 μg of rabbit polyclonal anti-PKCα antibody. The immunocomplex was recovered with protein-A sepharose 4B (50 μl slurry/lysate, Zymed Laboratory, South San Francisco, CA) and washed with the lysis buffer containing 0.4 M NaCl. The precipitated proteins were dissolved in 2× Laemmli's sample buffer [1× = 12 mM Tris·HCl, pH 6.8, 5% glycerol (v/v), 0.4% SDS (v/v), 2.88 mM 2-mercaptoethanol, and 0.2% bromphenol blue (v/v)], electrophoresed on a 10% polyacrylamide gel, transfered to polyvinylidene difluoride membrane, and immunostained with the same antibody using Vecstain ABC kit (Vector Laboratory, Burlingame, CA) and Konica immunostaining HRP-1000 (Konica, Tokyo, Japan).

Radiolabeling of phospholipids in intact cells. The phospholipid production by cells was measured according to the methods described by Traynor-Kaplan *et al.* (20). Cells were cultured to ~50% confluence in six-well plates and deprived of serum for 24 hr. During the last 3 hr of serum deprivation, the cells were incubated in phosphate-free RPMI1640 supplemented with ^{32}Pi (0.2 mCi/ml), washed, and then incubated in serum-free F-12 supplemented with 5 mM HEPES, pH 7.4. After incubation with wortmannin for 5 min or without incubation, the cells were stimulated with 10 nM ET-1 for 90 sec. The cells were then washed with ice-cold PBS and quenched by the addition of 10% (v/v) $HClO_4$. The cell lysate was transferred into a polypropylene tube, and after the addition of 4 ml of chloroform/methanol (1:2) and vigorous stirring, 2 ml of 10% $HClO_4$ and 2 ml of chloroform were added to separate the phases. One milliliter of the organic extract was dried under nitrogen, and the resulting pellet, dissolved in 10 μl of chloroform/methanol (95:5), was spotted onto a TLC plate (Silica Gel 60, Merck) that had been activated as described previously (20). The plate was developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7), dried, and then visualized for radioactivity with a BAS 2000 bioimaging analyzer. In every experiment, PI and PI(4)P were run on TLC as standards, and the lipid spots were visualized with iodine vapor.

Measurement of PI(3)K activity in cell lysates. Cells were grown to ~50% confluence in 100-mm plates, deprived of serum for 24 hr, washed with PBS, and then incubated in serum-free F-12 supplemented with 5 mM HEPES, pH 7.4. The reaction was started by the addition of ET-1 and terminated by aspiration of the medium and the addition of 1 ml of ice-cold lysis buffer [10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM Na orthovanadate, 1 mM AEBSF, 10 μg/ml aprotinin, and 10% (v/v) glycerol]. The reaction time was 90 sec. The cells were collected by scraping the dishes with a rubber policeman, lysed by a brief sonication, and then incubated on ice for 30 min. Insoluble material was removed by centrifugation at 10,000 × for 15 min. Twenty-five microliters of the lysate were mixed with 175 μl of reaction mixture consisting of 50 mM Tris·HCl, pH 7.4, 0.5 mM EGTA, 25 mM $MgCl_2$, 0.1 mM ATP, 1 μCi of [γ-^{32}P]ATP, and 0.2 mM PI(4,5)P_2 as a enzyme substrate. Where indicated, the cell lysate was incubated with wortmannin at 30° for 5 min before the addition of the reaction mixture. The reaction was incubated for 10 min at 30° and terminated by the addition of 50 μl of 8% (v/v) $HClO_4$ and 0.45 ml of chloroform/methanol (1:2). After vigorous shaking, the organic phase was separated by the addition of 0.25 ml of chloroform and 0.25 ml of 8% $HClO_4$, washed twice with chloroform-saturated solution containing 0.5 M NaCl and 1% $HClO_4$, and evaporated to dryness. The resulting pellet, dissolved in 10 μl of chloroform/methanol (95:5), was spotted onto a TLC plate (Silica Gel 60, Merck). The plate was developed in methanol/chloroform/25% NH_4OH/water (100:70:25:15), dried, and then visualized for radioactivity with a BAS 2000 bioimaging analyzer. In every experiment, PI(4)P and PI(4,5)P_2 were run on TLC as standards, and the lipid spots were visualized with iodine vapor.

Measurement of MAPK activity in cell lysates. Cells in 100-mm plates were processed exactly as described for the lysate preparations for the PI(3)K assay except that the stimulation time was 2 min and the lysis buffer was composed of 20 mM Tris·HCl, pH 7.4, 80 mM β-glycerophosphate, 2 mM DTT, 1 mM EGTA, 1 mM EDTA, 1 mM Na orthovanadate, 10 mM NaF, 1 mM AEBSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. MAPK was partially purified from the lysate through anion exchange chromatography according to procedures described previously (21). In brief, the lysate was applied to a DEAE-cellulose column (0.9 × 0.5 cm) pre-equilibrated with the lysis buffer. After the column was washed with 5 ml of lysis buffer containing 0.1 M NaCl, MAPK was eluted with 1 ml of lysis buffer containing 0.5 M NaCl. Analysis by SDS-polyacrylamide gel electrophoresis, Western transfer, and immunoblotting with monoclonal anti-MAPK antibody (Zymed Laboratory) verified complete recovery of MAPK (42- and 44-kDa species) in the 0.5 M NaCl eluate (data not shown). The protein concentration of the partially purified lysate was determined with the BCA microprotein assay kit, and the lysate was frozen at −80° until assay. Two assay procedures were used in the current study to measure the MAPK activity in the cell lysate. First, the kinase activity was determined with an assay kit (Amersham) according to the manufacturer's instructions. In brief, 15 μl of the cell lysate (2 μg of protein) was added to the same volume of a reaction mixture containing [γ-^{32}P]ATP (1 μCi/reaction) and a synthetic peptide (NH_2-KRELVEPLTPAGEAPNQALLR-COOH) as a substrate. The reaction was incubated at 30° for 30 min and terminated by the addition of a stop solution. The resulting solution was applied to a phosphocellulose membrane, and the membrane was washed extensively in 1% acetic acid and then in water. The radioactivity trapped on the membrane was measured with a liquid scintillation counter. Second, the kinase activity was measured with an in-gel kinase assay using MBP as a substrate as described previously (21). In brief, cell lysates (10 μg of protein) were resolved by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel containing 1 mg/ml MBP. The gel was washed with buffer A (50 mM HEPES, pH 7.4, containing 5 mM 2-mercaptoethanol) plus 20% of isopropanol at room temperature for 30 min. After denaturation in buffer A containing 6 N guanidine-HCl at room temperature for 1 hr and subsequent renaturation in buffer A with 0.04% Tween 40 at 4° for 16 hr, the gel was incubated at 25° for 1 hr in 10 ml of a reaction mixture (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 5 mM 2-mercaptoethanol, 50 μM ATP, and 250 μCi of [γ-^{32}P]ATP). The gel was extensively washed with 5% TCA containing 10 mM pyrophosphate. The bands of phosphorylated MBP were visualized with a BAS 2000 bioimaging analyzer.

Measurement of Raf-B kinase activity in cell lysates. Immunoprecipitation of Raf-B was done according to the procedures described previously (22). In brief, cells in 100-mm dishes were processed exactly as described for the lysate preparations for the PI(3)K assay except that the stimulation time was 2 min and the lysis buffer was composed of 20 mM Tris·HCl, pH 7.8, 137 mM NaCl, 5 mM EGTA, 1 mM EDTA, 2 mM DTT, 1 mM Na orthovanadate, 10 mM NaF, 1 mM

AEBSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 0.1 mM β-glycerophosphate, 10% (v/v) glycerol, and 1% (v/v) Triton X-100. Insoluble material was removed by centrifugation at 10,000 × *g* for 15 min. Immunocomplexes of Raf-B were prepared as described for immunoprecipitation of PKCα using 2 μg of antibody (Santa Cruz Biotechnology)/1 ml of lysate. The *in vitro* kinase activity of the recovered protein was assayed by the activation of MEK-1 in a coupled assay using recombinant MEK-1 and MAPK (p42) (Santa Cruz Biotechnology) as described previously (23). The immunocomplexes were washed twice with 1 ml of the lysis buffer and then twice with 1 ml of the kinase buffer without the substrate and ATP (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT). They were then incubated for 30 min at 30° in 20 μl of MAPK assay kit reaction mix (Amersham) containing MEK-1 (50 ng/reaction) and MAPK (50 ng/reaction). The subsequent procedures, including termination of the reaction and determination of the radioactivity incorporated in the substrate peptide, were exactly as described for the MAPK assay.

Statistical analysis. Where necessary, statistical analysis was done by analysis of variance.

Results

Mitogenic activity of ET-1 on CHO/ET_A cells. By co-transfecting cells (CHOK1, Ltk^-, and JH4) with pMEsf/ET_A and pSV*neo*^{r} constructs and then selecting for resistance against G418, we obtained more than three individual cell clones derived from each cell line that stably expressed ET_A. Binding assays with crude membrane preparations, ^{125}I-labeled ET-1, and unlabeled BQ123 (ET_A-specific antagonist) verified the expression of a single class of binding sites with B_{max} values of 0.5–4 pmol/mg protein and K_d values of 0.1–0.5 nM. Measurement of ET-1-induced $[Ca^{2+}]_i$ transients of Fura-2-loaded cells gave a similar pattern of the response regardless of the parental cell line; that is, (i) the response was biphasic, consisting of an initial robust peak followed by a sustained increase; (ii) maximal initial peak increments (0.8–1.2 μM) were obtained with 10 nM ET-1; and (iii) EC_{50} values for ET-1 to give half-maximum increments of the initial peak were ~1 nM. Despite the similar expression levels and $[Ca^{2+}]_i$ responses, initial screening with [^{3}H]thymidine incorporation assay revealed that only the transfectants derived from CHOK1 cells showed a significant increase in incorporation in response to ET-1. The data from a representative CHO/ET_A clone with a B_{max} value of 1.47 pmol/mg protein (which corresponds to ~250,000 receptors/cell) and a K_d value of 0.4 nM are shown in Fig. 1. Dose-response analysis gave EC_{50} values of 168 ± 14 pM and the maximum effect of 5.1 ± 0.2-fold increase (mean ± standard error, five experiments) above basal values. This increase in [^{3}H]thymidine incorporation was accompanied by cell cycle progression from G0/G1 to M phase (Fig. 1b) and by a dose-dependent increase in the cell number as revealed by MTT assay (Fig. 1a).

The conditioned medium from ET-1-stimulated (10 nM for 24 hr) CHO/ET_A cells failed to stimulate [^{3}H]thymidine incorporation in the parental CHOK1 cells (data not shown), excluding an involvement of any paracrine/autocrine factors in the mitogenic response. We also examined whether there was any synergism between the effect of ET-1 and that of classic growth factors. Of the four growth factors tested (IGF-1, bFGF, EGF, and platelet-derived growth factor-BB), IGF-1 (0.1–100 ng/ml) and bFGF (1–100 ng/ml) but not the other two factors caused dose-dependent increases in [^{3}H]thymidine incorporation and proliferation of CHO/ET_A cells. Coapplication of ET-1 (0.1–10 nM) caused no changes in the dose-dependent effects of either IGF-1 or bFGF, nor did coapplication of either IGF-1 (0.1–10 ng/ml) or bFGF (1–10 ng/ml) cause any changes in the dose-dependent effect of ET-1 (data not shown).

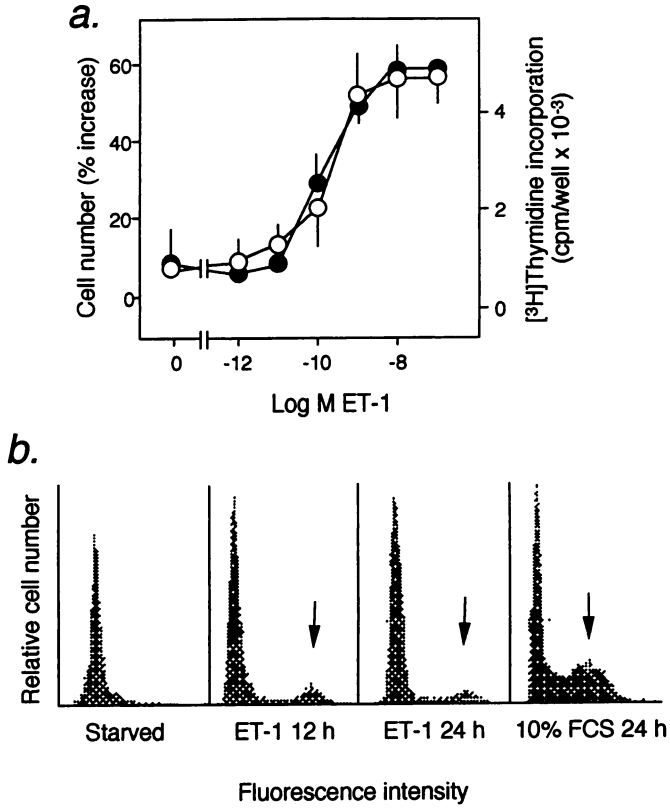

Fig. 1. ET-1-induced mitogenic responses of CHO/ET_A cells. a, Dose-dependencies for the effects of ET-1 on DNA synthesis and cell proliferation. Fasted cells in 96-well plates were stimulated with 10% FCS or with increasing concentrations of ET-1. [^{3}H]Thymidine incorporation at 24-hr stimulation (●) and the number of viable cells (MTT assay) at 48-hr stimulation (○) were determined as described in Experimental Procedures. The proliferation of the cells in the presence of ET-1 was expressed as relative to that in the presence of 10% FCS (100%). Shown are the mean ± standard error values of at least four determinations, each done in triplicate. b, Cell cycle analysis. Cells at 50% confluence in 100-mm plates were serum-deprived for 24 hr and then stimulated by 10 nM ET-1 or 10% FCS for the time indicated. Isolated nuclei were stained with propidium iodide and analyzed by flow cytometry. *Arrows*, peak for 4 N DNA.

Effects of U73122 and PMA treatment on ET-1-induced mitogenic responses of CHO/ET_A cells. ET_A couples with the $G_{\alpha q}$ subfamily of G_α proteins to activate PLC (24), and it is generally believed that the activation of PLC by $G_{\alpha q}$, which in turn leads to Ca^{2+} mobilization and PKC activation, is the main signaling pathway for $G_{\alpha q}$-coupled receptors. The fact that the ET-1-induced mitogenic responses were specific for CHO/ET_A and not shared by the other two cell lines, however, indicated that activation of Ca^{2+}/PKC signaling is not sufficient to induce the responses. The failure of PMA to induce an increase in [^{3}H]thymidine incorporation in CHO/ET_A cells (data not shown) supported this. To further explore the significance of Ca^{2+}/PKC signaling regarding the ET-1-induced mitogenic responses of CHO/ET_A cells, we tested the effects of the PLC inhibitor U73122 and the depletion of PKC by pretreatment of the cells with

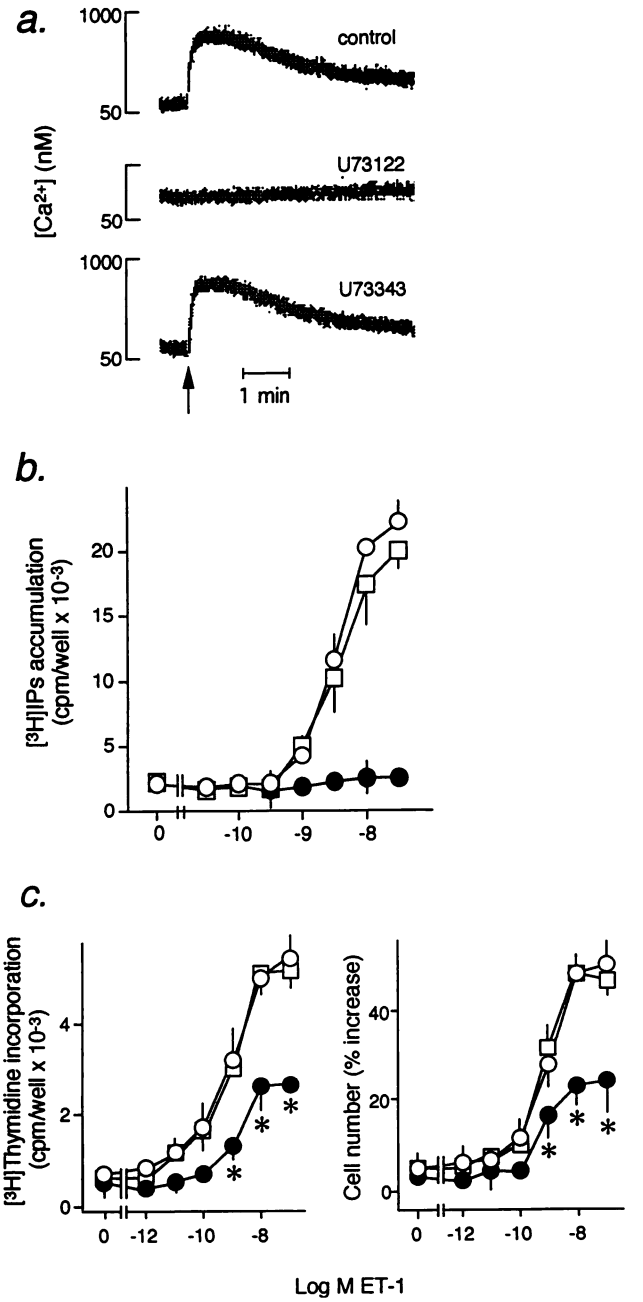

Fig. 2. ET-1-induced mitogenic responses of CHO/ET_A cells that are independent of PLC. a, Effects of U73122 on ET-1-induced increase in $[Ca^{2+}]_i$. Fura-2-loaded cells were stimulated with 10 nM ET-1 (*arrow*) in the presence or absence of 10 μM U73122 or its inactive analogue, U73343. The changes in $[Ca^{2+}]_i$ were monitored as described in Experimental Procedures. b, Effects of U73122 on ET-1-induced increase in [^{3}H]IP accumulation. Cells labeled with [^{3}H]inositol were stimulated with increasing concentrations of ET-1 in the absence (○) or presence of 10 μM U73122 (●) or U73343 (□). [^{3}H]IP accumulations were determined as described in Experimental Procedures. c, Effects of U73122 on ET-1-induced increases in DNA synthesis and cell proliferation. Fasted cells in 96-well plates were stimulated with increasing concentrations of ET-1 in the absence (○) or presence of 10 μM U73122 (●) or U73343 (□). [^{3}H]Thymidine incorporation at 24-hr stimulation (*left*) and the number of viable cells (MTT assay) at 48-hr stimulation (*right*) were determined as described in Experimental Procedures. Because U73122 caused no change in FCS (10%)-stimulated cell proliferation, the effect of ET-1 was expressed as relative to that of 10% FCS (100%). Shown are the mean ± standard error values of three determinations, each done in triplicate. *, $p < 0.01$, significantly different from the values in the absence of U73122.

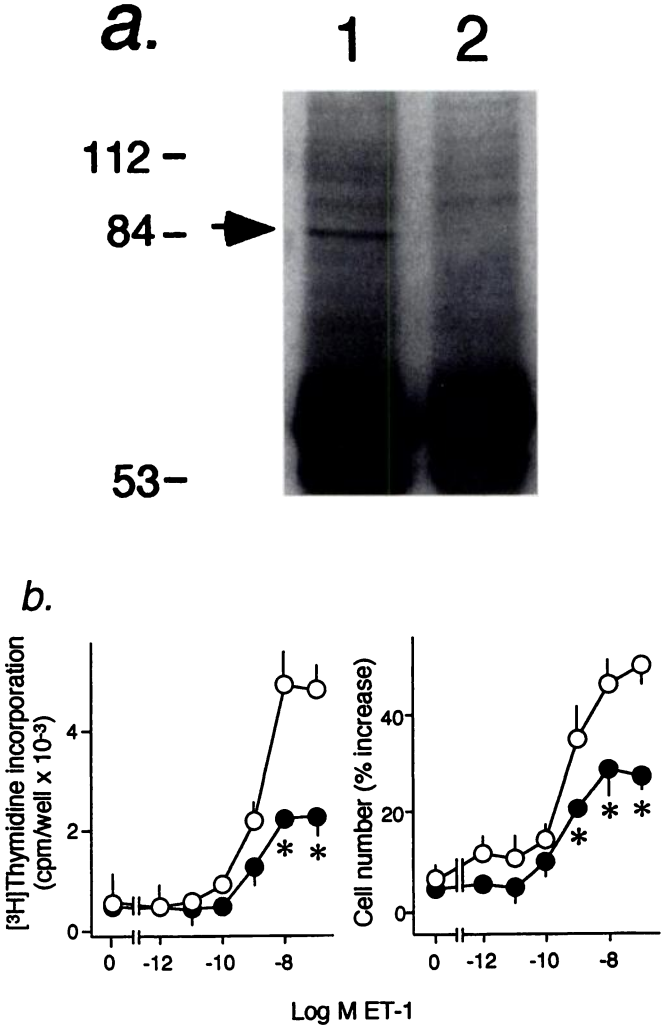

Fig. 3. ET-1-induced mitogenic responses of PMA-treated CHO/ET_A cells. a, Down-regulation of PKCα by PMA treatment. Cells in six-well plates were incubated for 24 hr in serum-free F-12 with (*lane 2*) or without (*lane 1*) 100 ng/ml PMA. PKCα was immunoprecipitated from the cell lysate and immunoblotted with anti-PKCα antibody as described in Experimental Procedures. *Numbers on the left* indicate the molecular masses in kDa. *Arrow* indicates the position of the specific band recognized by the antibody. The specificity of the band was verified by its disappearance in the presence of excess antigen peptide in the immunoblotting (not shown). b, Effects of PMA treatment on ET-1-induced increases in DNA synthesis and cell proliferation. Cells in 96-well plates were deprived of serum for 24 hr in serum-free F-12 with (●) or without (○) 100 ng/ml PMA. The cells were washed and then stimulated with increasing concentrations of ET-1. [^{3}H]Thymidine incorporation at 24-hr stimulation (*left*) and the number of viable cells (MTT assay) at 48-hr stimulation (*right*) were determined as described in Experimental Procedures. Because PMA treatment caused no change in FCS (10%)-stimulated cell proliferation, the effect of ET-1 was expressed relative to that of 10% FCS (100%). Shown are the mean ± standard error values of three determinations, each done in triplicate. *, $p < 0.01$, significantly different from the values of untreated cells.

PMA. When added before stimulation, U73122 (10 μM) completely inhibited both the ET-1-induced $[Ca^{2+}]_i$ response (Fig. 2a) and [^{3}H]IP accumulation (Fig. 2b). U73122 at the same concentration caused significant inhibition of the ET-1-induced increases in [^{3}H]thymidine incorporation and cell proliferation; the inhibition, however, was never complete (Fig. 2c). U73343, a structural analogue of U73122 that is inactive as a PLC inhibitor, failed to inhibit increases in

either $[Ca^{2+}]_i$ or [^{3}H]IP accumulation and had no effect on the mitogenic responses, suggesting that the observed effects of U73122 were caused by specific inhibition of PLC. PMA treatment of the cells (100 ng/ml for 24 hr) resulted in complete disappearance of PKCα, a representative of PKC (Fig. 3a). In PMA-treated cells, ET-1 still caused dose-dependent, significant increases in [^{3}H]thymidine incorporation and cell proliferation, although they were lesser in degree compared with its effects on control cells (Fig. 3b). These results suggested the presence of a mitogenic signaling pathway or pathways activated by ET_A that are independent of PLC.

Effects of wortmannin on ET-1-induced mitogenic responses of CHO/ET_A cells. Recently, two studies, one on guinea pig neutrophils (25) and the other on CHO cells expressing somatostatin receptors (26), revealed a Ca^{2+}/PKC-independent signaling pathway that linked PAF receptors and somatostatin receptors with MAPK, respectively. In both cases, the signaling was dependent on the activation of PI(3)K, which was inhibited by a fungal metabolite, wortmannin. Because the essential role of MAPK in mitogenesis has been well established, we tested the effect of wortmannin on the ET-1-induced mitogenic responses of CHO/ET_A cells. Wortmannin caused dose-dependent inhibition of the cell responses (Fig. 4a). The IC_{50} values for the inhibitory effects of wortmannin were within the range of 10–200 nM and depended on both the assays ([^{3}H]thymidine incorporation or cell proliferation) and the concentration of ET-1 applied. Wortmannin also caused dose-dependent inhibition of the ET-1-induced responses of PMA-treated cells (Fig. 4b), suggesting that the wortmannin-sensitive signaling was still activated by ET_A when PKC was down-regulated. When PMA-treated cells were stimulated by ET-1 in the presence of high concentrations ($\geq$1 μM) of wortmannin, there were virtually no mitogenic responses by the cells (Fig. 4b). The IC_{50} values for wortmannin inhibition of the effect of 10 nM ET-1 on PMA-treated cells were 31 ± 2 and 25 ± 4 nM (mean ± standard error, three experiments) for [^{3}H]thymidine incorporation and cell proliferation, respectively.

ET-1-induced activation of PI(3)K in CHO/ET_A cells. Because inhibition by wortmannin suggested the involvement of PI(3)K activity in the mitogenic signaling by ET_A, we tested whether ET-1 indeed caused an increase in PI(3)K activity. When ^{32}P-labeled cells were stimulated by ET-1 (10 nM), there was a large, rapid increase in the radioactivity incorporated into PIP_3 without an apparent change in the radioactivity incorporated into phosphatidylinositol bisphosphate, and a slight but significant increase in radioactivity incorporated into PIP (~3-fold increase by densitometry) (Fig. 5). The increase in PIP_3 formation was dose-dependently inhibited by the prior addition of wortmannin (Fig. 5), with IC_{50} values of 10–100 nM, suggesting the selective activation of PI(3)K elicited by ET-1. The activation of PI(3)K was confirmed by *ex vivo* experiments in which exogenous, purified $PI(4,5)P_2$ was used as an enzyme substrate and the formation of phosphatidylinositol-3,4,5-trisphosphate was measured (Fig. 6). ET-1 caused a dose-dependent increase in PI(3)K activity in the cell lysate, and the addition of wortmannin *in vitro* dose-dependently inhibited the activity, with IC_{50} values of ~30 nM.

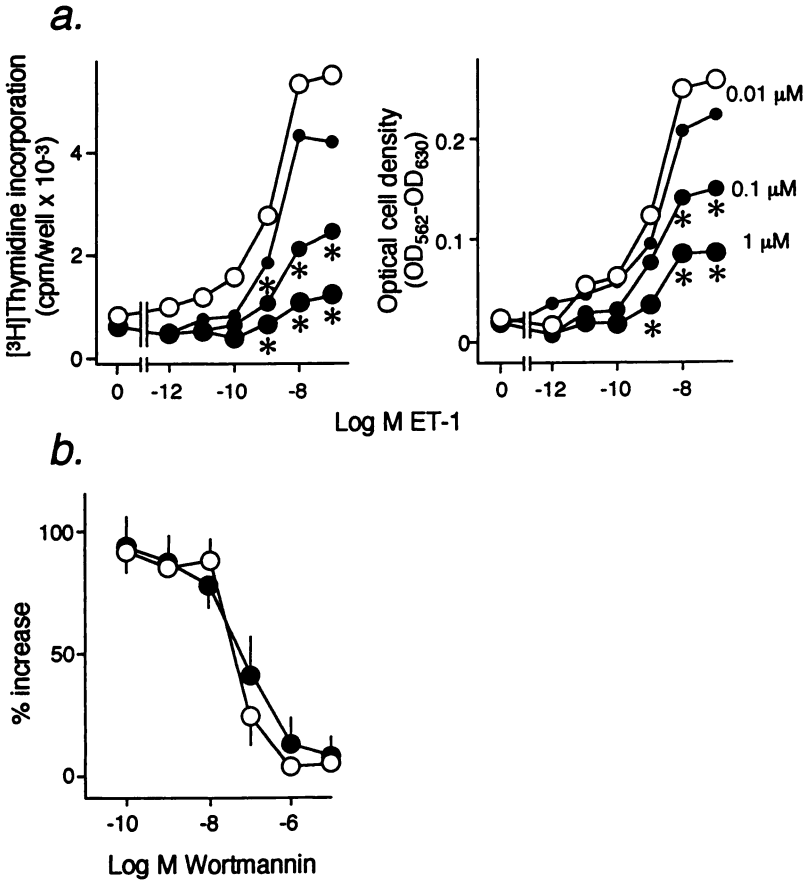

Fig. 4. Effects of wortmannin on ET-1-induced mitogenic responses of CHO/ET_A cells. a, Effects of wortmannin on ET-1-induced increases in DNA synthesis and cell proliferation. Fasted cells in 96-well plates were stimulated with increasing concentrations of ET-1 in the absence (○) or presence (●) of wortmannin at the concentrations indicated. [^{3}H]Thymidine incorporation at 24-hr stimulation (*left*) and the number of viable cells (MTT assay) at 48-hr stimulation (*right*) were determined as described in Experimental Procedures. Because wortmannin, at high concentrations ($\geq$100 nM), caused a significant decrease (~30%) in FCS (10%)-stimulated [^{3}H]thymidine incorporation and cell proliferation, the effect of ET-1 was expressed in absolute values. Shown are the mean of three determinations, each done in triplicate. For clarity, standard error values are not shown. *, $p < 0.01$, significantly different from the values of untreated cells. b, Effects of wortmannin on ET-1-induced increases in DNA synthesis and proliferation of PMA-treated CHO/ET_A cells. Fasted and PMA-treated (see legend to Fig. 3) cells were stimulated with 10 nM ET-1 in the absence or presence of increasing concentrations of wortmannin. [^{3}H]Thymidine incorporation at 24-hr stimulation (●) and the number of viable cells (MTT assay) at 48-hr stimulation (○) were determined as described in Experimental Procedures. Shown are the mean ± standard error values of three determinations, each done in triplicate.

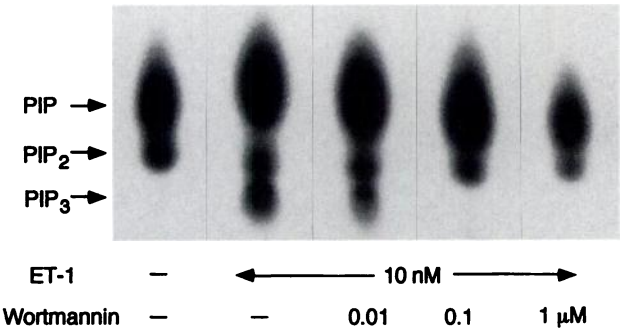

Fig. 5. Effect of wortmannin on ET-1-induced changes in ^{32}Pi incorporation into phosphoinositides in CHO/ET_A cells. Fasted and ^{32}Pi-labeled cells were incubated for 5 min with or without wortmannin at the concentrations indicated and then stimulated with 10 nM ET-1 for 90 sec. Phospholipids were extracted and separated by TLC together with standard phosphoinositides as described in Experimental Procedures. Shown is a portion of the autoradiogram that contains the positions of spots corresponding to phosphoinositides as indicated (*left*). Similar results were obtained for three independent experiments.

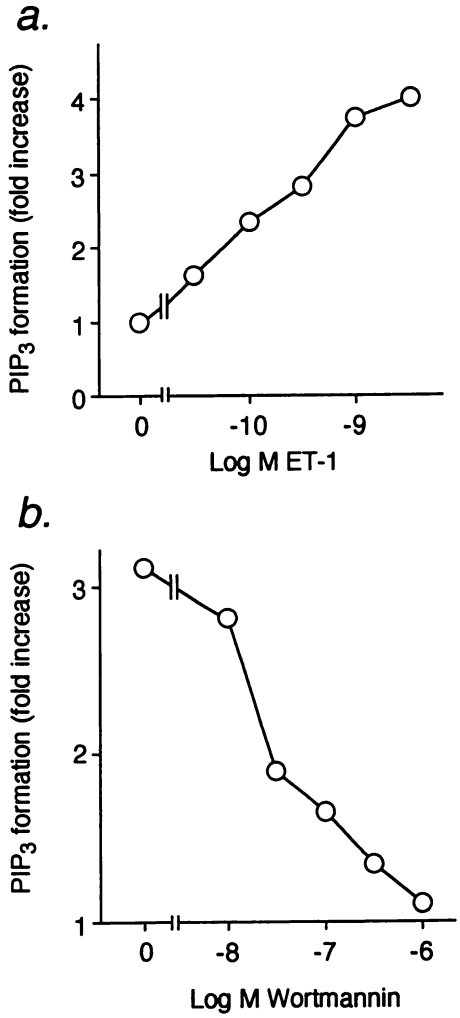

Fig. 6. ET-1-induced activation of PI(3)K in the cell lysate. a, Dose response for the effect of ET-1. Fasted cells in six-well plates were stimulated with increasing concentrations of ET-1 for 90 sec. The PI(3)K activity in the cell lysate was measured with partially purified $PI(4,5)P_2$ as a substrate as described in Experimental Procedures. b, Inhibition of PI(3)K activity by wortmannin *in vitro*. The lysate was prepared from the cells stimulated by ET-1 (3 nM) for 90 sec. It was incubated for 5 min at 30° with increasing concentrations of wortmannin, mixed with the reaction mixture, and then processed as described in Experimental Procedures. In both a and b, the density of spots on TLC plates corresponding to PIP_3 was analyzed with a BAS 2000 bioimaging analyzer and expressed relative to the control values. Shown are the representative results obtained in a single experiment. Similar results were obtained for two other independent experiments.

Effect of wortmannin on ET-1-induced activation of MAPK. PI(3)K has been suggested to be involved in the signaling from PAF or somatostatin receptor to MAPK (25, 26). To determine whether it was also involved in ET_A-activated signaling, we tested the effect of wortmannin on ET-1-induced MAPK activation in CHO/ET_A cells. Immunoblotting with monoclonal anti-MAPK antibody (Zymed Laboratory) detected two species of MAPK (42 and 44 kDa) in the cell lysate (data not shown), and ET-1 caused dose-dependent increases in the activities of both isoforms of MAPK (Fig. 7a). In accordance with the results of [^{3}H]thymidine incorporation and cell proliferation assays, ET-1 caused a significant increase in MAPK activity in PMA-treated cells and in the presence of U73122 (Fig. 7b), suggesting the presence of Ca^{2+}/PKC-independent signaling that linked ET_A with MAPK. Wortmannin, when applied before stimulation, caused dose-dependent inhibition of the effect of ET-1 but not of the effect of PMA on MAPK activity (Fig. 7c). Wortmannin also inhibited the effect of ET-1 on PMA-treated cells, and as in the case of [^{3}H]thymidine incorporation and cell proliferation assays, the inhibition was apparently complete when a high concentration (1 μM) of wortmannin was applied (Fig. 7d). The IC_{50} values for wortmannin inhibition of the effect of 10 nM ET-1 on PMA-treated cells were 55 ± 3 nM (mean ± standard error, three experiments). Wortmannin did not affect the expression levels of either species of MAPK, as revealed by immunoblotting analysis (data not shown), nor did it cause any changes in MAPK activity when added to the reaction mixture *in vitro* (data not shown).

Effects of wortmannin on ET-1-induced activation of Raf-B. The MAPK cascade includes MAPK, MAPK kinase (MEK), and MAPK kinase kinase (Raf and MEKK). The negative effect of wortmannin on PMA-stimulated MAPK activation suggested that the signaling pathway for PMA, i.e., PKC-Raf/MEKK-MEK-MAPK (27), is spared in the presence of the drug. To demonstrate it directly, we tested the effect of wortmannin on ET-1/PMA-stimulated activation of Raf-B in CHO/ET_A cells. Raf-B was chosen because it is the predominant form of the Raf family isoforms (Raf-1, Raf-A, and Raf-B) expressed by the cells.[3] We found that incubation of the cells with wortmannin (300 nM for 5 min) resulted in a significant decrease in the effect of ET-1 (10 nM for 2 min) on the kinase, whereas it barely affected the effect of PMA (100 ng/ml for 2 min) (Fig. 8). ET-1 induced Raf-B activation in PMA-treated cells, and this effect was also inhibited by wortmannin (Fig. 8). Wortmannin did not affect the expression levels of Raf-B as revealed by immunoblotting analysis (data not shown), nor did it cause any changes in Raf-B activity when added to the reaction mixture *in vitro* (data not shown).

[3] H. Ninomiya, unpublished observations.

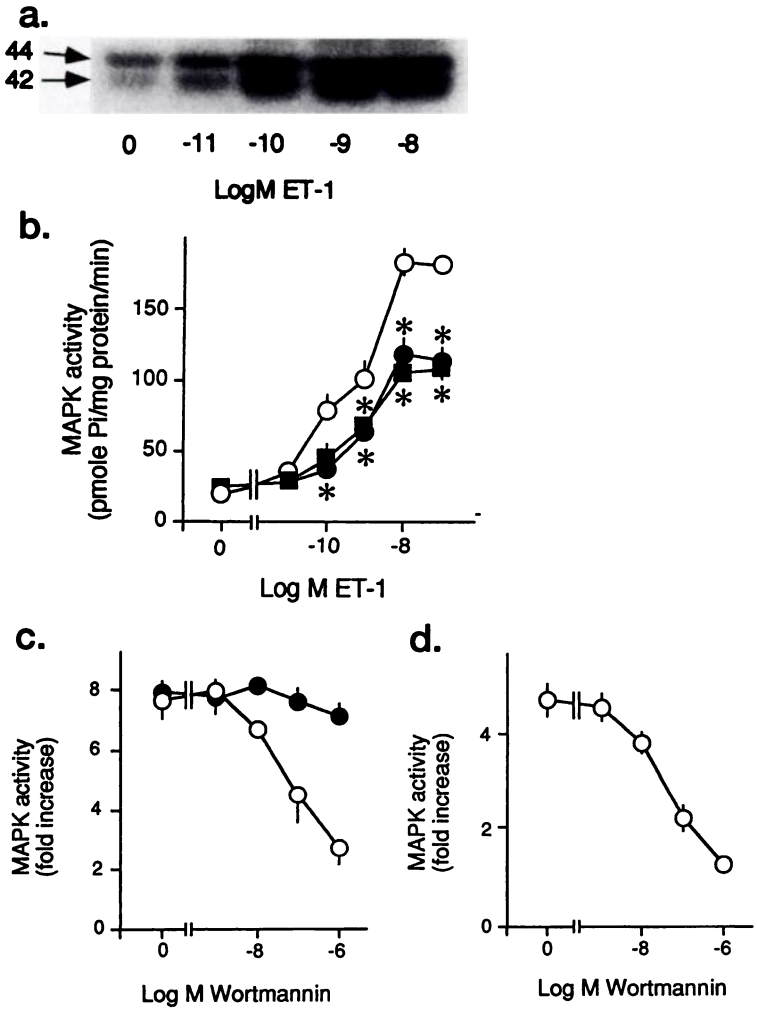

Fig. 7. ET-1-induced MAPK activation via a wortmannin-sensitive signaling pathway. a, Activation of 42- and 44-kDa species of MAPK. Fasted cells in six-well plates were stimulated with increasing concentrations of ET-1 for 2 min, and the MAPK activity in the cell lysate was measured with an in-gel kinase assay as described in Experimental Procedures. *Left*, molecular masses of the phosphorylated bands indicated in kDa. b, Ca^{2+}/PKC-independent activation of MAPK by ET-1. Fasted cells in six-well plates were stimulated with increasing concentrations of ET-1 for 2 min in the absence (○) or presence (●) of 10 μM U73122. In other sets of experiments, cells were fasted for 24 hr in serum-free medium containing 100 ng/ml PMA and then stimulated with ET-1 (■). In every case, the MAPK activity in the cell lysate was measured with an assay kit (Amersham). Shown are the mean ± standard error values of three determinations, each done in duplicate. *, $p < 0.01$, significantly different from the values of PMA-untreated cells in the absence of U73122. c, Inhibition of ET-1-induced, but not PMA-induced, activation of MAPK by wortmannin. Fasted cells in six-well plates were incubated for 5 min with or without increasing concentrations of wortmannin and then stimulated with either 10 nM ET-1 (○) or 100 ng/ml PMA (●) for 2 min. d, Inhibition of ET-1-induced activation of MAPK in PMA-treated cells by wortmannin. Cells fasted in the presence of PMA were incubated for 5 min with or without increasing concentrations of wortmannin and then stimulated with 10 nM ET-1 for 2 min. In both c and d, the MAPK activity in the cell lysate was measured with an assay kit (Amersham). Shown are the mean ± standard error values of three determinations, each done in duplicate. Basal MAPK activities were 26 ± 2 and 28 ± 3 pmol Pi/mg protein/min in PMA-untreated (c) and -treated (d) cells, respectively.

Discussion

In the case of ET_A, Ca^{2+}/PKC signaling is an ubiquitous response in every cell line expressing ET_A, and it is generally accepted that it is the main signaling pathway that leads to the activation of MAPK (4, 22, 27). Indeed, PKCα, a representative of PKC, has been shown to directly phosphorylate and activate Raf-1, which is one of the MAPK kinase kinases (27). The inhibition of MAPK activation (Fig. 7) and late mitogenic responses of the CHO/ET_A cells (Figs. 2 and 3) by either U73122 or PMA treatment underscores the role of this signaling pathway. In either case, however, the inhibition was never complete, suggesting the presence of an additional, PLC-independent link between ET_A and MAPK.

Results of the current study indicated the role of a wortmannin-sensitive signaling pathway as one of the PLC-independent mitogenic signaling pathways activated by ET_A. It has been reported that some G protein-coupled receptors (i.e., PAF and formyl-Met-Leu-Phe receptors) (28, 29) as well as tyrosine kinase receptors for many classic growth factors can elicit a rapid accumulation of phosphatidylinositol-3,4,5-trisphosphate, a major product of PI(3)K. In addition to the well-characterized PI(3)K regulated by tyrosine kinase receptors that contain SH2 domains in the regulatory subunit, the existence of a distinct PI(3)K activity regulated by G proteins has been suggested (30). The G protein-regulated PI(3)K was pharmacologically distinguished from the tyrosine kinase receptor-regulated PI(3)K by its diminished sensitivity to wortmannin, and a direct association of the enzyme with $\beta\gamma$ subunit was proposed as a possible activation mechanism for this type of PI(3)K (30). Recently, a G protein-activated PI(3)K with a potential pleckstrin homology domain was identified by molecular cloning, and this isotype was determined to be activated not only by $\beta\gamma$ subunit but also by the GTP-bound form of G_α subunit *in vitro* (31). In the current study, we showed that the activation of ET_A resulted in an increase of PI(3)K activity both *in vivo* (Fig. 5) and *in vitro* (Fig. 6). Although direct evidence is lacking, it is reasonable to assume the presence of the G protein-activated PI(3)K isoform or isoforms in CHO/ET_A cells and their activation by ET_A. That we could detect the activation of the kinase in the cell lysate (Fig. 6) suggested that some modification of the enzyme was caused by ET_A activation. If we assume that the activation of the enzyme was caused by a direct association with G protein subunit, one possibility is that the association was sufficiently tight to survive the lysing process. Further study is required to determine whether the *ex vivo* activation of the enzyme was due to any association with other proteins or to modification of the enzyme.

After the two reports (25, 26) that suggested the role of a wortmannin-sensitive pathway that linked G protein-coupled receptors (PAF or somatostatin receptors) and MAPK, we showed that a similar pathway linked ET_A and MAPK.

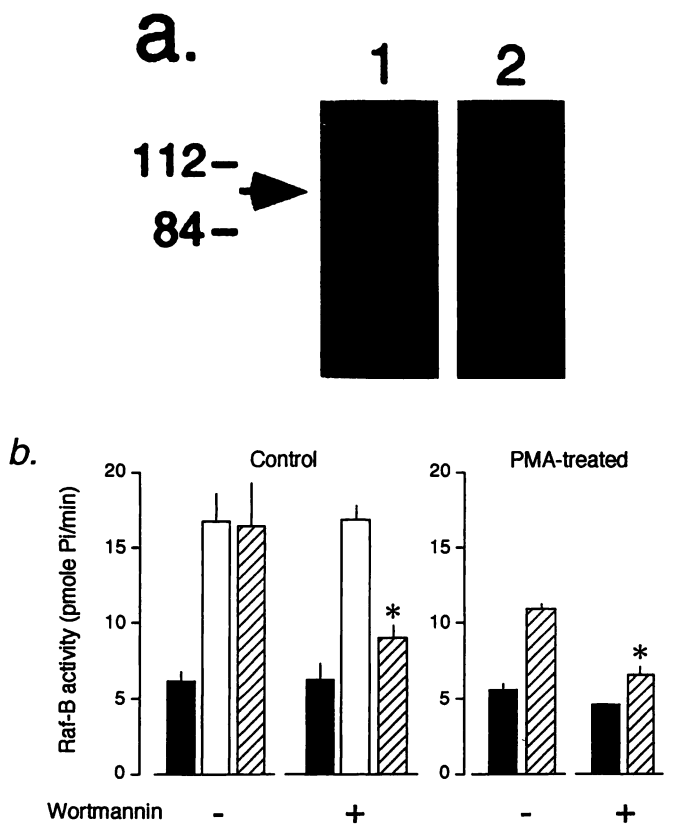

Fig. 8. Effects of wortmannin on ET-1-induced activation of Raf-B a, Immunocoprecipitation of Raf-B. Raf-B was immunoprecipitated from the cell lysate and analyzed by immunoblotting with anti-Raf-B antibody (*lane 1*). *Left*, molecular masses in kDa. *Arrow*, position of the band recognized by the antibody. The specificity of the band was verified by its disappearance in the presence of excess antigen peptide in the immunoblotting (*lane 2*). Shown are the blots of samples from unstimulated cells. PMA/ET-1 stimulation caused no change in the expression level of the proteins (not shown). b, Effects of wortmannin on ET-1/PMA-induced activation of Raf-B. Cells were fasted for 24 hr in serum-free medium with or without PMA (100 ng/ml). After incubation with or without 300 nM wortmannin for 5 min, they were unstimulated (*filled bars*) or stimulated for 2 min with either 10 nM ET-1 (*striped bars*) or 100 ng/ml PMA (*open bars*). The cell lysates were processed for Raf-B immunocomplex kinase assay as described in Experimental Procedures. Shown are the mean ± standard error values of three determinations. *, $p < 0.01$, significantly different from the values in the absence of wortmannin. PMA failed to induce Raf-B activation in PMA-treated cells (not shown).

Because previous studies did not examine the mitogenic responses of the cells, this was the first report that demonstrates the significance of the G protein-activated, wortmannin-sensitive signaling pathway to MAPK in the cells' mitogenic responses. There is an apparent difference between PAF/somatostatin receptors and ET_A in the G protein selectivity; both PAF and somatostatin receptors couple with pertussis toxin-sensitive G_α protein ($G_{\alpha i}$), whereas, at least in CHO/ET_A cells, ET_A does not couple with $G_{\alpha i}$ (18). Pertussis toxin treatment of CHO/ET_A cells failed to inhibit ET-1-induced increases in [^{3}H]thymidine incorporation and cell proliferation or activation of MAPK.[3] Thus, it is clear that the activation of $G_{\alpha i}$ is not necessary to activate the wortmannin-sensitive pathway that leads to MAPK activation.

Studies on tyrosine kinase receptors have indicated a number of signaling pathways activated by PI(3)K. The putative downstream mediators of PI(3)K include such diverse molecules as c-Akt (32, 33), some isoforms of PKC (34, 35), $p70^{S6K}$ (36), and low molecular weight G proteins (37, 38). Results of several studies on tyrosine kinase receptors (23, 39, 40), the current study, and other studies on G protein-coupled receptors (25, 26) have suggested that MAPK may also be the downstream mediator of PI(3)K. We found a good correlation between the effects of wortmannin on MAPK activity (Fig. 7) and mitogenic cell responses (Fig. 4), especially when PKC was down-regulated by PMA treatment, suggesting that MAPK is the major downstream mediator of PI(3)K in inducing mitogenesis. The current study, however, did not exclude the involvement of other effector molecules such as $p70^{S6K}$, which is important in translational regulation. Also unknown is the signaling pathway from PI(3)K to MAPK. We found an inhibitory effect of wortmannin on ET-1-induced activation of Raf-B (Fig. 8), suggesting the presence of a signaling pathway from PI(3)K to this kinase. This pathway is independent of PKC as demonstrated by the negative effect of wortmannin on PMA-induced activation and by the effect of ET-1 on PMA-treated cells (Fig. 8). This is the first observation of the inhibitory effect of wortmannin on mitogen-induced activation of Raf-B. In the literature, conflicting results of the effects of wortmannin on the Raf-1 kinase activity have been reported: Cross *et al.* (23) found an inhibitory effect of wortmannin on IGF-1/insulin-induced activation of the kinase in a skeletal/muscle cell line L6, whereas Karnitz *et al.* (40) reported a negative effect on IL-2-induced activation in T cell line CTLL-2. Although it is not known whether these discrepancies are simply due to the cells and stimulants used, we suggest that, at least in some systems, both PKC-dependent and PI(3)K-dependent pathways may lead to Raf activation. An interesting subject for future study is whether there is a difference among the Raf family isoforms and other MEK kinases in sensitivity to PI(3)-dependent pathway.

Of the ET_A transfectants obtained from three different cell lines (CHO, Ltk^-, and JH4), we found that only the clones derived from CHO cells exhibited late mitogenic responses ([^{3}H]thymidine incorporation and cell proliferation) in response to ET-1. Regardless of the parental cell line, every transfectant exhibited $[Ca^{2+}]_i$ response and MAPK activation (data not shown), indicating that these immediate responses are not sufficient to induce late mitogenic responses in some cell types. Similar dissociation between MAPK activation and [^{3}H]thymidine incorporation in response to ET-1 has been reported (41). Possible explanations for the dissociation include the dependence of the growth of each cell line on the duration of the signaling to MAPK (16) or on the signaling other than to MAPK.

In addition to its selective activity as a mitogen on CHO/ET_A cells, we demonstrated that ET-1 could act as a "full" mitogen on these cells, i.e., that ET-1 alone could induce cell proliferation and that no synergism was observed between ET-1 and two classic growth factors, bFGF and IGF-1. This observation is rather exceptional, and there have been many studies demonstrating an action of ET-1 as a "partial" mitogen or a "competent" factor in inducing mitogenic responses of the cells (4). For example, a synergism was reported between IGF-1 and ET-1 to induce DNA synthesis of Swiss 3T3 cells (23); a low concentration of platelet-derived growth factor or insulin was required for ET-1 to be effective on vascular SMCs (42, 43); and ET-1 also potentiated EGF-induced

transformation of Rat 1 or NRK49F cells (44, 45). One clear difference between CHO/ET_A cells and these cell types with endogenous expression of ETRs is the number of receptors; the receptor density (~250,000 sites/cell) expressed on a representative CHO/ET_A clone was ≥10 times higher than that expressed on fibroblasts or SMCs (21, 23). Thus, the differential effects of ET-1 as a mitogen might be simply due to the difference in receptor densities expressed by cells. In almost all previous studies, however, the functional expression of ETR was verified only by monitoring the ET-1-induced $[Ca^{2+}]_i$ response. The role of a wortmannin-sensitive pathway in the mitogenic signaling of ET-1 and its independence of Ca^{2+}/PKC-signaling in CHO/ET_A cells led us to propose an alternative hypothesis to explain the differential effects of ET-1: in a given cell type, the sensitivity of the wortmannin-sensitive pathway that involves PI(3)K may determine whether ET-1 acts as a "full" mitogen. The validity of the hypothesis will be tested in a future study.

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