Endothelin-1 Stimulates Contraction of Rat Glomerular Mesangial Cells and Potentiates β-Adrenergic-mediated Cyclic Adenosine Monophosphate Accumulation

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Abstract

The newly isolated peptide, endothelin-1 (ET-1), is a potent pressor agent that reduces GFR and the glomerular ultrafiltration coefficient. Recent evidence demonstrates that ET-1 mobilizes intracellular Ca^{2+} ($[Ca^{2+}]_i$) in glomerular mesangial cells by activating the phosphoinositide cascade. The present experiments were designed to examine whether ET-1 stimulates mesangial cell contraction and regulates the synthesis of PGE_2 and cAMP, which dampen vasoconstrictor-induced mesangial contraction. ET-1 (≥ 1 nM) reduced the cross-sectional area of rat mesangial cells cultured on three-dimensional gels of collagen type I. ET-1 also caused complex rearrangements of F-actin microfilaments consistent with a motile response. Contraction in response to ET-1 occurred only at concentrations that activate phospholipase C, and contraction was unaffected by blockade of dihydropyridine-sensitive Ca^{2+} channels. Elevation of $[Ca^{2+}]_i$ with ionomycin, to equivalent concentrations of $[Ca^{2+}]_i$ achieved with ET-1, also reduced mesangial cell cross-sectional area. ET-1 (0.1 μM) also evoked [^{3}H]arachidonate release and a fivefold increase in PGE_2 synthesis as well as increased synthesis of $PGF_{2\alpha}$ and small changes of TXB_2. ET-1 caused a minor increase in intracellular cAMP accumulation only in the presence of 3-isobutyl-1-methylxanthine. ET-1 also amplified cAMP production in response to isoproterenol. TPA and ionomycin, alone and in combination, failed to mimic the potentiating effect of ET-1; however, indomethacin blocked ET-1-induced potentiation of isoproterenol-stimulated cAMP, which was restored by addition of exogenous 10 nM PGE_2. Thus the present data demonstrate that ET-1 stimulates mesangial cell contraction via pharmacomechanical coupling and activates phospholipase A_2 to produce PGE_2, $PGF_{2\alpha}$, and TXB_2. ET-1 also amplified β adrenergic-stimulated cAMP accumulation by a PGE_2-dependent mechanism. (*J. Clin. Invest.* 1990. 85:790–797.) endothelium-derived mediators • prostaglandins • phospholipase A_2 • adenylate cyclase • vasoconstriction

Introduction

Endothelin-1 (ET-1)[1] is a member of a family of peptides synthesized by endothelial and other cell types in vitro and in vivo (1–3). Endothelin peptides arise from at least three preproendothelin species, which after proteolysis yield different isoforms (ET-1, -2, and -3, [1–3]).[2] ET-1 contracts diverse smooth muscle preparations in vitro (1, 3, 4), and when injected in vivo ET-1 is a potent vasoconstrictor (1, 3), particularly in the renal vasculature (5–10).

Recent experiments demonstrate a role for ET-1 in the control of glomerular hemodynamics. Miller et al. (8) and King et al. (9) found that ET-1 decreased both GFR and renal plasma flow; a disproportionate decline in renal plasma flow caused the filtration fraction to fall. Using micropuncture, Badr and co-workers (10) documented increased glomerular afferent and efferent arteriolar resistance and a dramatic decline in the ultrafiltration coefficient, K_f, in response to ET-1. Although the precise mechanism controlling K_f remains uncertain, the currently favored hypothesis is that contraction of mesangial cells, which are glomerular microvascular pericytes, decreases K_f by reducing filtration surface area (see 11–13 for review). We previously reported that ET-1 evokes two distinct patterns of Ca^{2+} signaling in cultured rat mesangial cells (14). ET-1 at 0.1–10.0 pM caused a modest but sustained elevation of $[Ca^{2+}]_i$ that depended on Ca^{2+} entry. In contrast, higher doses of ET-1 (0.1–100.0 nM) caused a rapid, transient increase in $[Ca^{2+}]_i$ followed by a lower, sustained phase (14). The transient phase of $[Ca^{2+}]_i$ correlated with activation of phospholipase C and the release of inositol-1,4,5-trisphosphate, which mobilizes Ca^{2+} from intracellular stores (15). These data implicate activation of the phosphoinositide cascade as an initial event leading to endothelin-induced biological changes, possibly including contraction of mesangial cells via pharmacomechanical coupling.

Using cultured rat mesangial cells, the present experiments were designed to examine whether ET-1 causes mesangial cell contraction and stimulates synthesis of two negative feedback signals, prostaglandin E_2 (PGE_2) and cAMP, which dampen mesangial contraction (11, 12, 16). Our experiments provide evidence that ET-1 (*a*) stimulates mesangial contraction which correlates with activation of the phosphoinositide cascade; (*b*) increases [^{3}H]arachidonate release and PGE_2, $PGF_{2\alpha}$, and TXB_2 synthesis; and (*c*) amplifies β-adrenergic-stimulated cAMP accumulation via a PGE_2-dependent mechanism.

Methods

Materials. Porcine ET-1 was from Peninsula Laboratories (Belmont, CA). Bovine dermal collagen type I was purchased from Collagen

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1. *Abbreviations used in this paper:* AVP, arginine vasopressin; DHP, dihydropyridine; DPBS, Dulbecco's PBS; IBMX, 3-isobutyl-1-methylxanthine; TPA, 12-tetradecanoyl phorbol 13-acetate.

2. The nomenclature for designating endothelin isoforms is that suggested by Drs. Masashi Yanigasawa and Tomah Masaki at a recent Endothelin Workshop held at the William Harvey Research Institute, London, England in December 1988. ET-1 refers to the 21 amino acid porcine and human sequence.

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Corp. (Palo Alto, CA). Arginine vasopressin (AVP), 12-tetradecanoyl phorbol 13-acetate (TPA), ionomycin, nifedipine, and Bay K 8644 were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Rhodamine-phalloidin and fura-2 acetoxymethylester were from Molecular Probes (Grand Junction, OR). [5,6,8,9,11,12,14,15-^{3}H]Arachidonate (85 μCi/mmol) was from New England Nuclear (Boston, MA). SQ29,548 was from Squibb Pharmaceuticals (Princeton, NJ). All other biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Mesangial cell culture and contraction assays. Rat mesangial cell strains in passages 5–15, cultured and characterized as reported (17), were subcultured onto three-dimensional, type I collagen gels in Lab-Tek (Nunc) culture slides for measurements of cell contraction. Type I collagen gels were prepared by mixing 8.0 ml of 2.9 mg/ml collagen in 0.012 N HCl, 1.0 ml of 1 N NaOH, and 1.0 ml of 10× Minimal Essential Medium, all from sterile solutions at 4°C, and adjusting to pH 7.4. Aliquots (1 ml) were plated into two-chamber Lab-Tek slides and gelation was initiated by warming to 37°C for 1 h. Contraction was tested 24 h after subculture onto the collagen gel by incubating cells plus or minus agonists in Hanks' balanced salt solution containing 1.6 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM Hepes (pH 7.4), and 2 mg/ml fatty acid-free bovine serum albumin (HBSS-II), and the change in cross-sectional area over time was measured using computer-aided microscopy as previously described (17). A cell was scored positive for contraction with a decrease in cross-sectional area ≥ 7%. The significance of responding cells was tested by Chi-squared analysis (18).

Staining of F-actin microfilaments. 24 h after subculture into four-chamber Lab Tek slides, mesangial cells were incubated with HBSS-II alone or with agonists for 30 min at 23°C. To evaluate cytoskeletal arrangements, cells were rinsed, twice, with Dulbecco's PBS (DPBS), fixed with 0.5 ml 3.7% formaldehyde in DPBS for 10 min at 23°C, rinsed, twice, with DPBS, permeabilized with 0.5 ml 0.2% Triton X-100 in DPBS (vol/vol) for 1 min at 23°C, and rinsed again, three times, with DPBS. Cells were then treated with 0.2 ml 5.0 U/ml rhodamine-phalloidin for 30 min at 23°C on a rotary shaker in the dark. After rinsing, twice, with DPBS, cells were mounted under glycerol/DPBS (1:1), sealed, and photographed using a Leitz microscope with a mercury lamp, epifluorescence illumination, and Kodak TMax film. The photomicrographs in Fig. 3 were taken with identical exposures and the prints developed under identical conditions so that semiquantitative comparisons of relative staining intensities can be made.

Measurements of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined with the Ca^{2+}-sensitive dye fura-2 (19). As reported (14, 20), confluent monolayers on plastic Aclar coverslips (Allied Engineered Plastics, Pottsville, PA) were loaded with 1 μM fura-2 acetoxymethylester for 40 min at 37°C in RPMI 1640, then washed, twice, and incubated again for 20 min in fura-2 free RPMI to allow for intracellular dye cleavage. The coverslips were mounted in a quartz cuvette with 2 ml of Krebs-Henseleit Hepes buffer, pH 7.4, maintained at 37°C with constant stirring. $[Ca^{2+}]_i$ was determined by measuring fluorescence with a University of Pennsylvania Biomedical Instruments Group spectrofluorimeter set at 339 nm excitation and 500 nm emission. Fluorescence measurements were converted to Ca^{2+} concentrations by determining maximal fluorescence (F_{max}) with 10–40 μM ionomycin, followed by minimal fluorescence (F_{min}) with 7.5 mM EGTA, 60 mM Tris, pH 10.5 (14, 20). The following formula was then used: $[Ca^{2+}]_i = K_d (F\text{-}F_{min}/F_{max}\text{-}F)$, K_d for fura-2 = 224 nM (19). Autofluorescence by the cells or agonists was negligible.

[^{3}H]Arachidonate release and prostaglandin synthesis. For measuring [^{3}H]arachidonate release, cells in six-well plates were incubated for 20 h with 1 μCi/ml [^{3}H]arachidonate in RPMI 1640 medium with 17% fetal bovine serum. Incorporation into cellular lipid was ≥ 85% of added [^{3}H]arachidonate. The labeling media was aspirated and the cells washed, three times, at 5-min intervals with HBSS-II. After the final wash agonists were added and 0.2-ml aliquots of buffer were removed without replacement at the indicated times. Counts were corrected for volume, and counts at time 0 were subtracted; all conditions were measured in duplicate wells. For measurements of prostaglandin synthesis, subconfluent monolayers in 12-well plates (4 cm^2/well) were washed, twice, with 1 ml RPMI 1640 medium. Incubations were in 0.5 ml RPMI 1640 at 37°C in 5% CO_2/95% air for 15 min, after which the supernates were immediately collected and frozen at −40°C. All conditions were in duplicate. Cellular protein was measured by the method of Bradford (21). Immunoreactive PGE_2, $PGF_{2\alpha}$, and TXB_2 were measured by RIA as previously described in this laboratory (17). All compounds used were checked independently for interference in the assays. Eicosanoid biosynthesis was corrected for total cellular protein per well and expressed as nanograms per milligram protein/15 min.

Measurements of intracellular cAMP accumulation. Mesangial cell monolayers in 12-well plates were washed, twice, with RPMI 1640 medium, 10 mM Hepes (pH 7.4). At time 0, agonists were added in the same medium, or where indicated with 0.1 mM 3-isobutyl-1-methylxanthine (IBMX). All conditions were in duplicate. After incubating for 3 min at 37°C, the supernates were discarded and cyclic nucleotides extracted in 0.1 N HCl for 3 h at 25°C (22). The extracts were stored frozen at −40°C. Cyclic AMP was detected, after acetylation, by RIA (20) and all compounds checked for interference with cAMP-antisera binding. Cellular protein was measured as described above and cAMP accumulation was expressed as fmol/μg protein/3 min.

Results

ET-1 reduces cross-sectional area of mesangial cells. We assessed the contractile actions of ET-1 using mesangial cells cultured on three-dimensional collagen gels. When ET-1 was added at 0.1 μM, 48% of cells demonstrated a marked reduction of cross-sectional area at 30 min (arrows in Fig. 1, Table I). Cross-sectional area in the responding cells fell by an average of 42%, but in some cells reductions of 60–70% were seen. Contraction most often occurred along the long axis of the cell, although in many cells the cell body was also reduced (Fig. 1). Only 4.3% of control cells (i.e., HBSS-II alone) showed a significant (i.e., > 7%) reduction in cross-sectional area (Fig. 1, Table I). 1 pM ET-1, which generates only a monophasic, slow but sustained elevation of $[Ca^{2+}]_i$ (14), failed to induce mesangial cell contraction (Table I). In contrast, two concentrations of ET-1 (1.0 nM and 0.1 μM) that mobilize intracellular Ca^{2+} via activation of phospholipase C (14) increased the percentage of contracted cells above control (Table I). Elevation of mesangial cell $[Ca^{2+}]_i$ with 1 and 10 μM ionomycin, to similar concentrations of $[Ca^{2+}]_i$ mobilized by 0.01 and 0.1 μM ET-1 (Fig. 2 and ref. 14), also reduced mesangial cell cross-sectional area in 25% and 67% of the cells, respectively (Table I). Contraction in response to ET-1 was unaffected by 10 μM nifedipine, which blocks dihydropyridine (DHP)-sensitive Ca^{2+} channels. Further evidence that ET-1-induced contraction occurs independent of DHP-sensitive Ca^{2+} channels comes from experiments with Bay K 8644, a synthetic DHP Ca^{2+} channel agonist. Although Bay K 8644 stimulated a slow, sustained increase in $[Ca^{2+}]_i$ in mesangial cells (Fig. 2), Bay K 8644 was unable to evoke a decrease in cross-sectional area in a significant percentage of mesangial cells compared to control incubations in HBSS-II (Table I). These data are consistent with ET-1-induced contraction of mesangial cells via pharmacomechanical coupling (i.e., activation of phospholipase C) and fail to support a role for DHP-sensitive Ca^{2+} channels.

As shown in Fig. 3, we examined the arrangement of actin microfilaments in mesangial cells treated with ET-1 and ionomycin (Fig. 3). In control cells incubated for 30 min in HBSS-II alone, F-actin was assembled in long, linear microfilament bundles distributed evenly throughout the cytosol along the

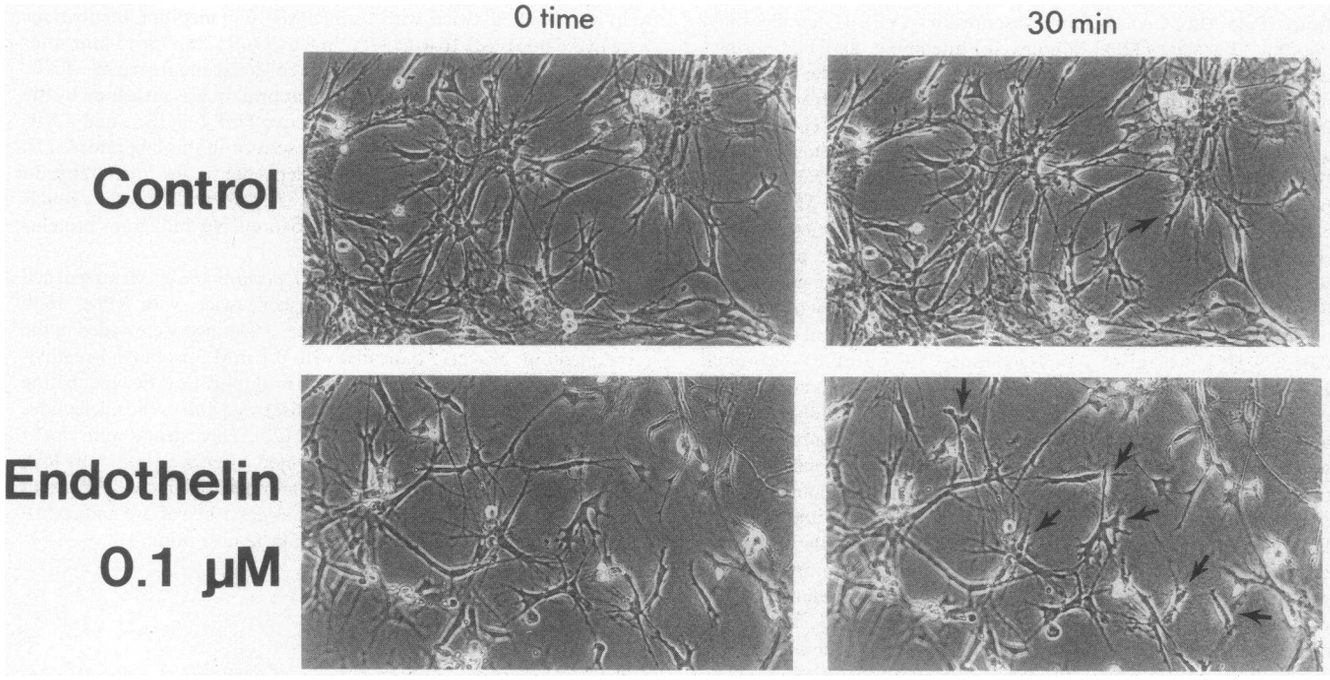

Figure 1. ET-1 stimulates contraction of rat glomerular mesangial cells. As described in Methods, mesangial cells were subcultured onto three-dimensional type I collagen gels, and 24 h later (time 0) the medium was aspirated and replaced with HBSS-II minus or plus 0.1 μM ET-1 for 30 min at 25°C. The cross-sectional area of individual cells was measured (17) at time 0 and 30 min later, and any cell exhibiting a decrease in cross-sectional area ≥ 7% at 30 min vs. time 0 was scored positive for contraction. Arrows identify contractile cells. Similar results were observed in five separate experiments in different mesangial cell strains. Table I summarizes the contraction data. ×85.

long axis of the cell (Fig. 3, *A* and *B*). These bundles resembled thick stress fibers. In > 60% of cells treated with 0.1 μM ET-1 or 10 μM ionomycin, actin microfilaments assumed a diffuse, irregular meshwork state (Fig. 3, *C, E, F*) typical of motile cells. In general the amount of F-actin stainable with rhodamine-phalloidin was increased, even in the larger, flattened cells that failed to retract (Fig. 3 *D*). There were localized areas of intense condensation of actin microfilaments (single arrows, Fig. 3, *C, E, F*), particularly in the retracted cells. Many cells treated with ET-1 or ionomycin elaborated membrane-associated condensations of F-actin that formed bundles parallel to the plasma membrane (double arrows, Fig. 3, *C, E, F*). These rearrangements of actin microfilament bundles in mesangial cells incubated with ET-1 and ionomycin are consistent with an agonist-induced transition from stationary cells expressing stress fibers to motile cells elaborating a diffuse meshwork organization.

Table I. Reduction of Cross-sectional Area in Response to ET-1 by Mesangial Cells on Collagen Gels

Effector	Total cells	Responding cells	Chi-squared analysis
	n	*n (%)*	
Control	47	2 (4)	—
ET-1			
1.0 pM	21	0 (0)	NS
1.0 nM	38	8 (21)	$P \leq 0.05$
0.1 μM	65	31 (48)	$P \leq 0.01$
Ionomycin			
1.0 μM	16	4 (25)	$P \leq 0.05$
10.0 μM	15	10 (67)	$P \leq 0.01$
ET-1, 0.1 μM + nifedipine, 10 μM	37	16 (43)	$P \leq 0.01$
Bay K 8644, 50 μM	34	2 (6)	NS

The reduction of cross-sectional area by individual mesangial cells was measured during a 30-min incubation with the indicated effectors and inhibitors as described in Methods. Any cell that reduced its cross-sectional area by ≥ 7% at 30 min was scored positive for contraction and considered a responding cell (17). Data are from three to four separate experiments, and chi-squared analysis compared the significance of responding vs. nonresponding cells for each condition (18).

ET-1 caused release of [^{3}H]arachidonate and synthesis of arachidonate metabolites. To test whether ET-1 stimulates release of arachidonic acid from membrane phospholipids, ET-1 was added to mesangial cells equilibrium labeled with [^{3}H]arachidonate. ET-1 (0.1 μM) evoked a time-dependent increase in [^{3}H]arachidonate release which plateaued by 10 min (Fig. 4 *A*). By 10 min ~ 0.3–0.5% of incorporated [^{3}H]arachidonic acid was released by ET-1. Both the time course and magnitude of [^{3}H]arachidonate release in response to ET-1 were similar to [^{3}H]arachidonate release in the presence of 1.0 μM AVP. ET-1 also increased production of immunoassayable PGE_2, $PGF_{2\alpha}$, and TXB_2 (Fig. 4 *B*). ET-1 at 0.1 μM generated a fivefold increase in PGE_2 synthesis; by comparison, 0.1 μM AVP stimulated a 2.7-fold increase. Activation of protein kinase C by TPA has been reported to amplify phospholipase A_2 activity in cultured mesangial cells (23). In the present experiments, 0.1 μM TPA alone doubled PGE_2 production, and TPA had neither an additive nor a synergistic effect on ET-1- or AVP-stimulated PGE_2 production. That 0.1 μM TPA was

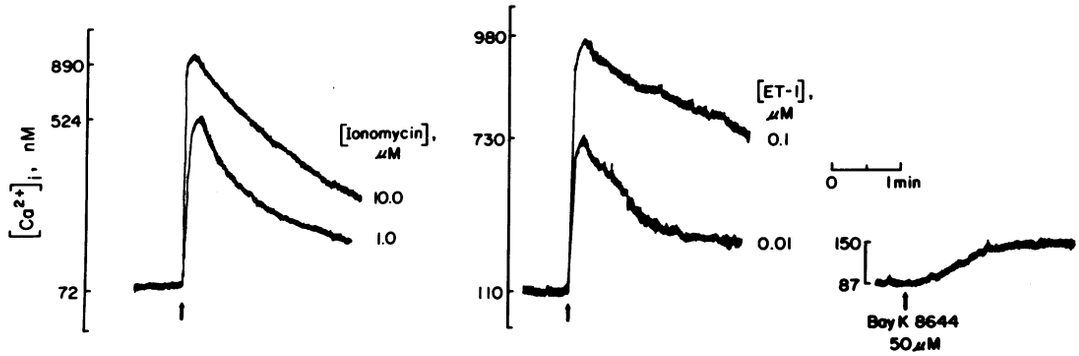

Figure 2. Ionomycin-, ET-1-, and Bay K 8644-induced changes in $[Ca^{2+}]_i$. Fura-2-loaded mesangial cell monolayers on plastic coverslips were suspended in Krebs-Henseleit Hepes buffer, pH 7.4; basal $[Ca^{2+}]_i$ was measured after thermal equilibrium at 37°C. Using different monolayers, at the indicated times (*arrows*) 1.0 or 10 μM ionomycin, 0.01 or 0.1 μM ET-1, or 50 μM Bay K 8644, were added to the rapidly stirred cuvettes. Tracings of ionomycin-induced $[Ca^{2+}]_i$ were superimposed to facilitate comparison of the waveforms. The $[Ca^{2+}]_i$ waveforms evoked by 1.0 and 10 μM ionomycin approximated the $[Ca^{2+}]_i$ signal induced by 0.01 and 0.1 μM ET-1, respectively (14). Identical responses were observed for each condition in four different monolayers in separate experiments.

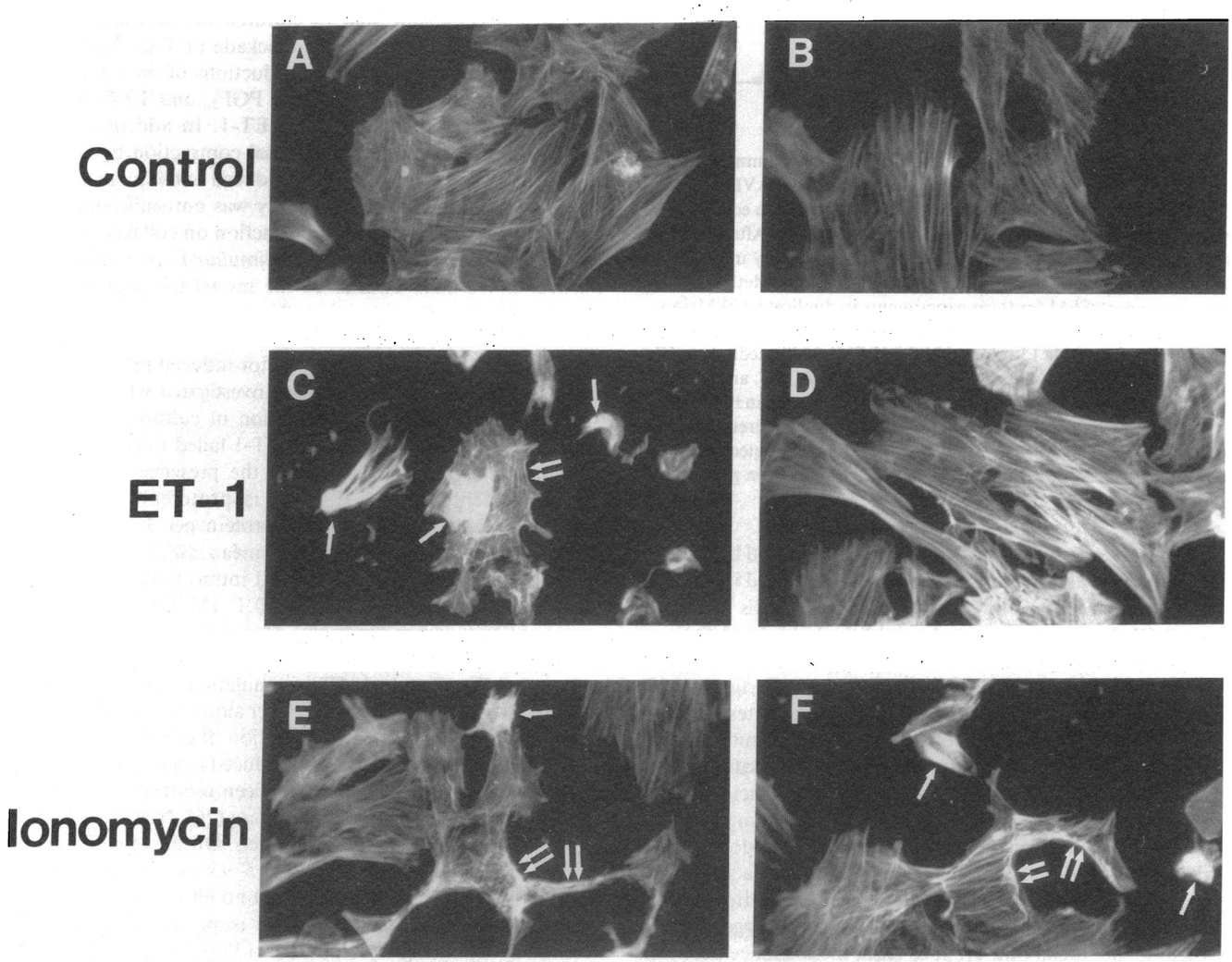

Figure 3. ET-1 and ionomycin cause complex rearrangements of F-actin microfilament bundles in mesangial cells. Cells on glass chamber slides were treated with HBSS-II (*A, B*), 0.1 μM ET-1 (*C, D*), or 10 μM ionomycin (*E, F*) as described in Fig. 1. The cells were then fixed in formaldehyde, permeabilized with Triton X-100, stained with rhodamine-phalloidin, and photographed under fluorescence microscopy as described in Methods. Specific properties of cell structure identified by arrows are described in the text. ×160.

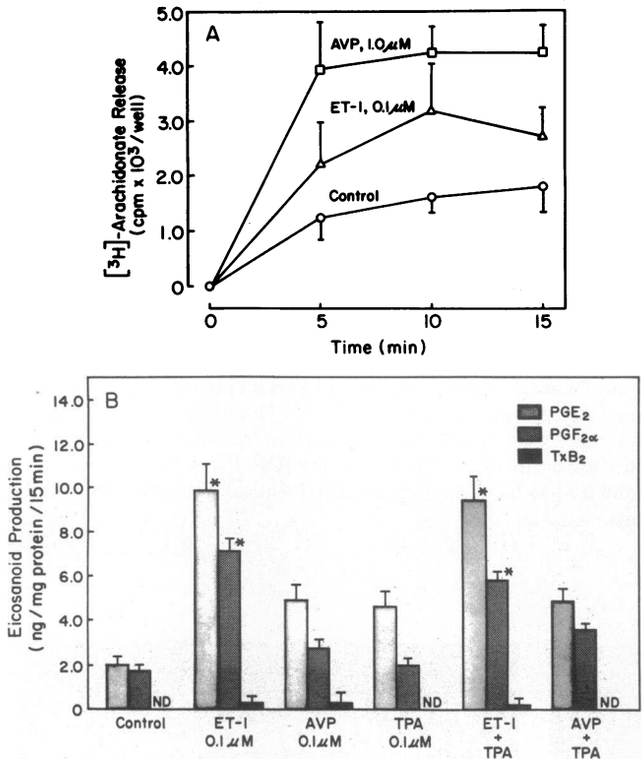

Figure 4. Release of [^{3}H]arachidonate and synthesis of immunoreactive arachidonate metabolites in response to ET-1 and AVP. (*A*) Mesangial cells at 80% confluency in six-well plates were equilibrium-labeled for 20 h with 1 μCi/ml [^{3}H]arachidonate. After washing the cells, agonists were added and release of radioactivity into HBSS-II was measured at the times indicated as described in Methods. Data are mean±SEM for three experiments in duplicate. (*B*) Mesangial cells at 80% confluency in 12-well plates were incubated with the effectors indicated for 15 min at 37°C in RPMI 1640 medium in 5% CO_2/95% air. Release of immunoreactive PGE_2, $PGF_{2\alpha}$, and TXB_2 into the supernate was quantified by RIA. Data are mean±SEM for four to five experiments in duplicate. *Significantly different from control values ($P < 0.05$) by Student's *t* test. ND, not detectable with a minimum detectable limit of 0.05 ng TXB_2/mg protein per 15 min.

sufficient to activate protein kinase C was suggested by concurrent experiments where TPA stimulated a threefold increase in [^{3}H]thymidine uptake by quiescent mesangial cells incubated with 0.5% fetal bovine serum (unpublished observations).

PGE_2 is thought to attenuate mesangial cell contraction whereas $PGF_{2\alpha}$ and TXB_2 have contractile properties (11–12, 16). To determine if the arachidonate metabolites resulting from ET-stimulated phospholipase A_2 activity could mediate or regulate ET-induced contraction, we preincubated mesangial cells in the contraction assay with indomethacin to block cyclooxygenase and with SQ29,548, a thromboxane receptor blocker. A 10-min preincubation with 10 μM indomethacin, which blocks ≥ 95% of mesangial cyclooxygenase activity (24), modestly increased the percentage of cells exhibiting a significant decline in cross-sectional area (Table II), suggesting that arachidonate metabolites reduce basal mesangial cell contractility. Indomethacin, however, did not influence the reduction in cross-sectional area induced by 0.1 μM ET-1 (Table II). Preincubation with 10 μM SQ29,548 likewise had no effect in either control incubations or in incubations with ET-1 (Table II). Thus we conclude that in cultured rat mesangial cells, inhibition of cyclooxygenase or blockade of TXA_2 receptors have no effect on ET-1-induced reductions of mesangial cell cross-sectional area, suggesting that $PGF_{2\alpha}$ and TXB_2 do not mediate contraction induced by ET-1. In addition, even though indomethacin amplified basal contraction by mesangial cells, the amount of PGE_2 produced subsequent to ET-stimulated phospholipase A_2 activity was not sufficient by itself to dampen ET-1-induced contraction on collagen gels.

Table II. Effect of Indomethacin and SQ29,548 on the Reduction in Mesangial Cell Cross-sectional Area Induced by ET-1

Effector	Total cells	Responding cells	Chi-squared analysis
	n	*n* (%)	
Control	24	2 (8)	—
Indo, 10 μM → medium	29	5 (17)	$P \leq 0.05$
Indo → ET-1, 0.1 μM	54	24 (44)	$P \leq 0.01$
SQ29,548, 10 μM → medium	24	3 (13)	NS
SQ29,548 → ET-1	26	13 (50)	$P \leq 0.05$

Mesangial cells on type I collagen gels were incubated for 10 min with 10 μM indomethacin (Indo) or 10 μM SQ29,548 before the addition of medium alone or 0.1 μM ET-1. The cells were then incubated for an additional 30 min, and the change in mesangial cell cross-sectional area during the entire 40-min incubation was measured as described in Methods. Data are from two to four separate experiments. indo, indomethacin.

ET-1 potentiates β adrenergic-stimulated accumulation of intracellular cAMP. Similar to the model for regulation of smooth muscle cell contraction (25), the concentration of intracellular cAMP is thought to be an important negative-feedback signal to dampen vasoconstrictor-induced mesangial cell contraction (11–13, 16). Thus we investigated whether ET-1 would stimulate cAMP accumulation in cultured mesangial cells. When added alone, 0.1 μM ET-1 failed to elevate intracellular cAMP (Fig. 5). Added in the presence of 0.1 mM IBMX, which magnifies 1.0 μM isoproterenol-stimulated cAMP 6.2-fold (189±17 fmol/μg protein per 3 min without IBMX vs. 1,169±67 with IBMX, mean±SEM, n = 4–6 in duplicate), ET-1 modestly increased intracellular cAMP over basal levels (basal = 40±5 vs. 0.1 μM ET-1 = 85±10, mean±SEM, n = 5 in duplicate). However, coincubation with ET-1 (0.1 μM), in the absence of IBMX, nearly doubled isoproterenol-stimulated cAMP accumulation (Fig. 5). Ionomycin (10 μM) and TPA (0.1 μm), either alone or in combination, were unable to mimic the potentiation of cAMP synthesis by ET-1 (Fig. 5). Indeed ionomycin reduced isoproterenol-stimulated cAMP synthesis, which has been ascribed to Ca^{2+}-mediated activation of phosphodiesterase (26). Neither TPA nor ionomycin affected basal cAMP levels. On the other hand, 10 μM indomethacin, which inhibits > 95% of cyclooxygenase activity in mesangial cells (24), had no effect on basal cAMP levels but blocked potentiation of isoproterenol-stimulated cAMP by ET-1 (Fig. 5). Addition of PGE_2 to an extracellular concentration (10 nM) similar to that stimulated by 0.1 μM ET-1 (Fig. 4 *B*) mimicked the amplification of cAMP accumulation by ET-1. Similar amplification of cAMP accumulation was also observed when 0.1 and 1.0 nM PGE_2 were coin-

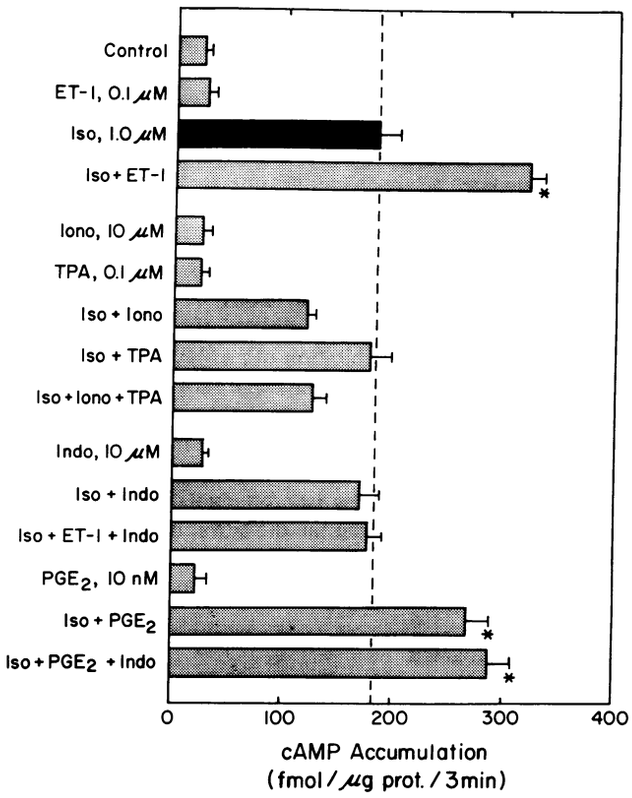

Figure 5. ET-1 amplifies isoproterenol-stimulated cAMP accumulation by a PGE_2-dependent mechanism. Mesangial cells at 80% confluency in 12-well plates were washed and incubated for 3 min at 37°C in RPMI 1640 medium with 10 mM Hepes, pH 7.4; IBMX was not used in these experiments. After extraction in 0.1 N HCl, radioimmunoassayable intracellular cAMP was measured after acetylation as described in Methods. The dashed line denotes cAMP accumulation in the presence of 1.0 μM isoproterenol alone. Data are mean±SEM for four to seven experiments in duplicate. Abbreviations: Iso, isoproterenol; Iono, ionomycin; Indo, indomethacin. *Significantly different from 1.0 μM isoproterenol values ($P < 0.01$) by Student's *t* test.

cubated with ET-1. Coincubation with isoproterenol, PGE_2 and indomethacin ruled out nonspecific interactions between adenylate cyclase and indomethacin. 10 nM PGE_2 is below the threshold for activation of adenylate cyclase (Fig. 5 and reference 24), demonstrating that direct stimulation of adenylate cyclase by PGE_2 did not account for the potentiating effect with isoproterenol. Thus these results suggest that an increase in $[Ca^{2+}]_i$ and activation of protein kinase C do not mediate ET-1's potentiation of cAMP accumulation. Instead these data support the hypothesis that ET-1-stimulated PGE_2 synthesis mediates amplification of β adrenergic-stimulated cAMP accumulation by ET-1.

Discussion

There is accumulating evidence that the biological functions of ET-1 are of considerable importance in the kidney. ET-1 markedly increases renal vascular resistance and decreases renal blood flow, glomerular filtration rate, and K_f (5–10). In the rat ET-1 at low doses modestly increases NaCl excretion (9) and decreases renin secretion from isolated glomeruli (27). In the dog ET-1 decreases NaCl excretion and increases plasma renin activity (8). We (14) and others (10) have shown that ET-1 is a potent mitogen for glomerular mesangial cells and that ET-1 evokes the phosphoinositide cascade. The present results demonstrate that ET-1 stimulates contraction of rat glomerular mesangial cells and increases PGE_2 synthesis, which in turn potentiates β adrenergic-stimulated cAMP accumulation. The increase in intracellular cAMP concentration could potentially dampen contraction in response to ET-1 to form a negative feedback loop (11, 25). Several lines of evidence suggest that ET-1-induced contraction is mediated by a Ca^{2+} signaling system activated by phospholipase C. First, contraction in response to ET-1 occurred only at concentrations (i.e., ≥ 1.0 nM, see Table I and reference 14) that evoke a $[Ca^{2+}]_i$ transient via activation of phospholipase C and the release of (1,4,5)IP_3. 1 pM ET-1, which generates only a modest increase of $[Ca^{2+}]_i$ independent of phospholipase C (14), failed to stimulate contraction. Second, when we mimicked the shape and magnitude of the ET-1-generated $[Ca^{2+}]_i$ waveform with ionomycin, equal or greater numbers of cells contracted than with ET-1 (Fig. 2, Table I). Third, the inability of nifedipine to attenuate contraction in response to ET-1 argues against a role for voltage-gated Ca^{2+} channels. Moreover, the minor $[Ca^{2+}]_i$ waveform in response to high concentrations of Bay K 8644 (Fig. 2), and the lack of contraction by cells incubated with Bay K 8644 (Table I), imply that under the present conditions mesangial cells express DHP-sensitive Ca^{2+} channels in low numbers or with diminished activity that are not functionally coupled to mesangial contraction. Using a different approach, Takeda and colleagues (28) reached similar conclusions regarding the role of voltage-gated Ca^{2+} channels in mesangial cell contraction in response to AVP. Fourth, staining with rhodamine-phalloidin revealed complex ET-1- and Ca^{2+}-mediated rearrangements of mesangial F-actin microfilament bundles. These rearrangements resemble the transition from stationary cells elaborating thick stress fibers to motile cells exhibiting a diffuse meshwork state of actin microfilaments. Also, intense condensations of F-actin were observed contiguous to and parallel to the plasma membrane (Fig. 3, *C, E, F,* double arrows) where Latta (29) and Sakai and Kriz (30) in vivo, and Ausiello et al. (31) in vitro, previously documented extensive microfilament bundles (which resemble actomyosin complexes) by electron microscopy. Thus the available data support the hypothesis that activation of phospholipase C and the subsequent mobilization of $[Ca^{2+}]_i$ by Ins 1,4,5-P_3 mediate ET-1-induced mesangial cell contraction.

In the present study, we employed mesangial cells cultured on three-dimensional, type 1 collagen gels to assess cell contraction. The use of a collagen substrate was prompted by reports showing that culture on or within type I collagen gels promotes expression of a contractile phenotype in isolated aortic myocytes (32) and bovine retinal microvascular pericytes (33). Mesangial cells on collagen gels expressed more abundant cytoplasmic extensions than cells on polystyrene, and these cells closely resemble the phenotype of mesangial cells in vivo (29, 30). In vivo these cytoplasmic extensions are dense with contractile filaments (29, 30) and are thought to play a vital function in the control of K_f by mesangial contraction (11–13, 16, 30). ET-1-induced contraction occurred in the cell body but was pronounced in the cytoplasmic extensions and along the long axis of the cell (Fig. 1). The improved contractile response of mesangial cells cultured on type I col-

lagen gels should facilitate investigations of mesangial contraction.

In the normal mesangium, PGE_2 and PGI_2, through stimulation of cAMP, help maintain renal function by counteracting the effects of vasoconstrictors on mesangial contraction (11–13, 16). Our experiments provide evidence for a novel mechanism by which ET-1 would elevate intracellular cAMP in mesangial cells. ET-1 stimulated [^{3}H]arachidonate release and PGE_2, $PGF_{2\alpha}$, and TXB_2 synthesis consistent with ET-1 activation of phospholipase A_2. Similar activation of phospholipase A_2 by ET-1 was found by De Nucci et al. (4) who recently demonstrated that ET-1 released PGI_2 and TXA_2 in guinea pig and rat isolated lungs. Experiments with indomethacin and SQ29,548 suggested that $PGF_{2\alpha}$ and TXB_2 do not mediate ET-1-induced mesangial contraction. Although the results with indomethacin support a role for PGE_2 in attenuating basal contractillity, ET-1 failed to stimulate sufficient accumulations of PGE_2 in culture to counteract the potent contractile activity of ET-1 (Table II). Likewise the concentration of PGE_2 produced ($\sim$ 10 nM) in mesangial cells was insufficient to activate adenylate cyclase above basal levels (Fig. 5). The threshold for activation of adenylate cyclase by PGE_2 is $\geq$ 100 nM in cultured mesangial cells (24). In contrast, in the present experiments ET-1 nearly doubled cAMP production by isoproterenol (Fig. 5). Agonists that trigger the phosphoinositide cascade have been reported to potentiate β adrenergic-stimulated cAMP accumulation by a protein kinase C-dependent pathway (26, 34–36), although the biochemical mechanism remains unclear. For example, Nakiba et al. (37) have found that in vascular smooth muscle cells, angiotensin II amplifies cAMP accumulation stimulated by isoproterenol and vasoactive intestinal peptide. But in mesangial cells, ionomycin and TPA, alone and in combination, failed to mimic amplification of cAMP synthesis (Fig. 5), suggesting that an increase in $[Ca^{2+}]_i$ or protein kinase C activity did not mediate the potentiating effect ET-1. These results are similar to studies in guinea-pig cerebral cortical slices, where TPA and ionomycin failed to simulate histamine's ability to potentiate adenosine-stimulated cAMP accumulation (38). In contrast, indomethacin blocked amplification of cAMP accumulation in mesangial cells by ET-1, and addition of exogenous 10 nM PGE_2 restored the potentiation (Fig. 5). Because this concentration of PGE_2 was insufficient to activate adenylate cyclase, the effect of PGE_2 was not additive but potentiating. This pathway for potentiating cAMP accumulation contrasts with the view that direct activation of adenylate cyclase by PGE_2 receptors accounts for the vasorelaxant properties of PGE_2 (16, 24). Thus it appears that PGE_2 can elevate intracellular cAMP in mesangial cells by two distinct pathways: (*a*) one pathway the result of direct activation of adenylate cyclase by PGE_2; (*b*) the other due to potentiation by PGE_2 of β adrenergic-stimulated cAMP accumulation. It is interesting to note that potentiation by ET-1 approximately doubled the intracellular concentration of cAMP in the absence of IBMX (Fig. 5), whereas to achieve a doubling by direct activation required the addition of > 10 μM exogenous PGE_2 even in the presence of IBMX (24). Given the well-documented role of cAMP to dampen smooth muscle cell contraction (25), it seems possible that amplification of β adrenergic-mediated cAMP accumulation by ET-1 could attenuate ET-1-induced contraction, but this hypothesis remains to be tested.

The biochemical mechanism by which PGE_2 amplifies β-adrenergic–stimulated cAMP accumulation is at present unclear. Similar results were observed by Siegel et al. (39) in human platelets where PGE_2 and PGI_2, at concentrations insufficient to directly activate adenylate cyclase, markedly potentiate forskolin-stimulated cAMP synthesis. PGE_2 and PGI_2 increase both the efficacy and potency of forskolin as an activator of adenylate cyclase. Amplification by PGE_2 of forskolin-stimulated cAMP synthesis has also been observed in brain slices (40, 41). Another possible mechanism would be inhibition of cAMP efflux by PGE_2, PGA_2, or PGA_1. In certain cell types cAMP efflux is inhibited by $PGA_1 = PGA_2 > PGE_2 \gg PGF_{2\alpha}$ (42–44), putatively via a glutathione conjugate of the prostaglandin (45). Thus inhibition of cAMP export by ET-1 could result directly from PGE_2 or from PGA_2 formed by the nonenzymatic hydrolysis of PGE_2. In the present experiments, the net effect of reduced cAMP efflux on the intracellular concentration of cAMP would be enhanced when adenylate cyclase activity was elevated by isoproterenol. Further experiments are necessary to determine the site(s) of action for the potentiating effect of ET-1-induced PGE_2 synthesis on β adrenergic-stimulated cAMP accumulation in mesangial cells.

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