Endothelin$_B$ Receptor Activates NHE-3 by a Ca^{2+}-dependent Pathway in OKP Cells

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Abstract

To examine the mechanisms by which endothelin (ET) regulates the Na/H antiporter isoform, NHE-3, OKP cells were stably transfected with ET_A and ET_B receptor cDNA. In cells overexpressing ET_B, but not ET_A receptors, ET-1 increased Na/H antiporter activity ($J_{Na/H}$). This effect was inhibited by a nonselective endothelin receptor blocker and by a selective ET_B receptor blocker but was not inhibited by an ET_A selective receptor blocker. In ET_B-overexpressing cells, 10^{-8} M ET-1 inhibited adenylyl cyclase, but protein kinase A inhibition and pertussis toxin pretreatment did not affect Na/H antiporter activation by ET-1. ET-1 caused a transient increase in cell [Ca^{2+}], followed by a sustained increase. Increases in cell [Ca^{2+}] were partially inhibited by pertussis toxin. ET-1–induced increases in $J_{Na/H}$ were 50% inhibited by clamping cell [Ca^{2+}] low with BAPTA, and by KN62, a Ca-calmodulin kinase inhibitor. Inhibitors of protein kinase C, cyclooxygenase, lipoxygenase, and cytochrome P450 and cyclic GMP were without effect. In ET_A-overexpressing cells, ET-1 increased cell [Ca^{2+}] but did not increase $J_{Na/H}$. In summary, binding of ET-1 to ET_B receptors increases Na/H antiporter activity in OKP cells, an effect mediated in part by increases in cell [Ca^{2+}] and Ca-calmodulin kinase. Increases in cell [Ca^{2+}] are not sufficient for Na/H antiporter activation. (*J. Clin. Invest.* 1996. 97: 1454–1462.) Key words: Na/H antiporter • proximal tubule • adenylyl cyclase • endothelin

Introduction

The endothelins (ET)[1] are potent vasoconstrictors that function as paracrine and autocrine factors. Three isoforms, ET-1, -2, and -3, interact with two receptors, ET_A and ET_B. ET_A receptors bind ET-1 and ET-2 with high affinity, whereas ET-3 binds with low affinity; ET_B receptors bind ET-1, -2, and -3 with similar high affinities (1, 2). In addition to causing vasoconstriction, the endothelins may also increase blood pressure through renal sodium retention. At low concentrations, ET-1 increases proximal tubule sodium and volume absorption, whereas at high concentrations these processes are inhibited (3). Proximal tubule transcellular NaCl absorption is mediated by an apical membrane Na/H antiporter, encoded predominantly by the isoform NHE-3 (4–6). ET-1 applied to renal cortical slices or directly to brush border membrane vesicles stimulates apical membrane Na/H antiporter activity (7, 8).

OKP cells express NHE-3 and have been useful for the study of NHE-3 regulation (9). In the present studies, we overexpressed ET_A and ET_B receptors in OKP cells and examined the regulation of NHE-3 by ET-1. The results demonstrate that binding of ET-1 to the ET_B receptor activates Na/H antiporter activity. This effect is mediated in part through increases in cell [Ca^{2+}] ([Ca^{2+}]$_i$) and activation of Ca-calmodulin (CaM) kinase.

Methods

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted as follows: penicillin and streptomycin from Whittaker M.A. Bioproducts (Walkersville, MD); culture media and G-418 from GIBCO BRL (Gaithersburg, MD); cAMP kit from Amersham Corp. (Arlington Heights, IL); (2′7′)bis (2-carboxyethyl)-5,6-carboxyfluorescein–acetoxymethyl ester (BCECF-AM), Fura-2-AM, Pluronic, and dimethyl BAPTA-AM from Molecular Probes (Eugene, OR); ET-1 from Peptides International (Louisville, KY); PTH from Calbiochem (San Diego, CA); H89, KN62, and H7 from LC Laboratories (Woburn, MA); and ^{125}I-ET-1 from Amersham (Arlington Heights, IL).

Cell culture. OKP cells were passaged in high glucose (450 mg/dl) DME supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). For study, cells were grown in low glucose (100 mg/dl) DME. When confluent, cells were rendered quiescent for 48 h before study by the removal of serum. To overexpress human ET_A and ET_B receptors, cells were transfected with plasmids pMEhET_A and pMEhET_B, which contained the respective receptor cDNAs driven by an SRα promoter (10, 11). Each plasmid was cotransfected with pSV2-neo using calcium phosphate coprecipitation. Neomycin-resistant cells were selected by growth in 400 μg/ml G-418 and maintained in 200 μg/ml G-418. Clonal cell lines were isolated by limiting dilution and screened by Northern blot for maximal expression. For experimentation, G-418 was removed at the time of splitting, 5–7 d before study.

To measure ET-1 binding, cells were grown to confluence in 12-well plates, rendered quiescent, and incubated in medium containing 0.3% BSA, 20 pM ^{125}I-ET-1, and varying amounts of cold ET-1 for 1 h at 37°C. Incubation medium was then aspirated, the cells were washed twice with cold PBS, and the cells were harvested in 1 N NaOH.

Measurement of intracellular pH and Na/H antiporter activity. Continuous measurement of cytoplasmic pH (pHi) was accomplished in cells grown on coverslips using the intracellularly trapped pH-sensitive dye BCECF, as previously described (12). pHi was estimated

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1. *Abbreviations used in this paper:* AM, acetoxymethyl ester; BCECF, (2′7′)bis(2-carboxyethyl)-5,6-carboxyfluorescein; [Ca^{2+}]$_i$, intracellular calcium concentration; CaM kinase, calcium calmodulin–dependent kinase; ET, endothelin; L-NA, *N*-nitro-L-arginine; NDGA, nordihydroguaiaretic; NO, nitric oxide.

from the ratio of fluorescence with excitation wavelengths of 500 and 450 nm and emission wavelength 530 nm (SLM 8000C, Rochester, NY). Calibration of the BCECF excitation ratio was accomplished using the nigericin technique as described (12).

Na/H antiporter activity was assayed as the initial rate of Na-dependent pHi recovery from an acid load in the absence of CO_2/HCO_3. For this assay, Na-containing solution was (in mM): 130 Na, 5.0 K, 1.1 Ca, 1.5 Mg, 140.2 Cl, and 30 Hepes. In Na-free solutions, Na was replaced with choline. All solutions were adjusted to pH 7.4 with *N*-methyl-D-glucammonium hydroxide at 37°C. Cells were first bathed in Na-containing solution, and baseline pHi was measured. The bath was then changed to Na-free solution containing 13 μM nigericin × 4 min, which caused pHi to decrease to ~ 6.4–6.6. Nigericin was then removed, and the cells were washed with 1% (wt/vol) dialyzed BSA in Na-free solution for 2 min. Cells were then bathed in the Na-free perfusate for 30 s. Subsequent replacement with Na-containing solution caused a rapid pHi recovery that was due to the Na/H antiporter (13). The initial rate of this Na-dependent pHi change (dpHi/dt) was calculated by drawing a tangent to the initial deflection. In all studies, control and experimental cells were from the same passage and were assayed on the same day. pHi at which Na was added was not different between groups. Exposure to ET-1 was begun at the time of loading with BCECF and was maintained throughout the duration of the assay (~ 35 min) unless otherwise stated. All control cells were exposed to appropriate vehicle.

To calculate buffer capacity, cells were pulsed with 10 mM NH_4Cl in the Na-free perfusate, and buffer capacity was calculated at the trough pHi, as previously described (14). 10^{-8} M ET-1 had no effect on buffer capacity in ET_B6 cells ($\beta = 40.4 \pm 1.9$ vs 42.2 ± 2.4 mM/liter · pH U, ET-1 vs control, NS). Na/H antiporter activity is therefore expressed as dpHi/dt.

cAMP radioimmunoassay. For measurement of cAMP production, cells were rinsed twice with PBS and incubated in 1 ml of serum-free medium at 37°C in 5% CO_2 for 1 h with or without ET-1 and with 10^{-7} M PTH and 2 mM 3-isobutyl-1-methylxanthine. Control wells were exposed to appropriate vehicles. After incubation, cell protein was precipitated by the addition of 100 μl of 100% TCA to each well. After 20 min at room temperature, the supernatant was carefully harvested, extracted three times with water-saturated ether, and used to measure cAMP concentration by radioimmunoassay. Protein content per well was determined after solubilization of the precipitate with 1 N NaOH, as described by Lowry et al. (15).

Cell calcium. To measure intracellular calcium concentration ($[Ca^{2+}]_i$), cells were suspended in PBS containing 2 mM EDTA by periodic shaking, washed in a Ca^{2+}-containing solution (in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 11 glucose, 10 Hepes, pH 7.4, 0.1% BSA), and incubated in 4 μM Fura-2-AM with 0.04% Pluronic for 35 min. Cells were then washed and resuspended in the Ca^{2+}-containing solution and maintained on ice until experimentation. Intracellular calcium was measured in cells suspended in Ca^{2+}-containing solution as the ratio of fluorescence with 340- and 380-nm excitation and 510-nm emission (fluorolog 2; Spex Industries, Edison, NJ). The fluorescent ratio was calibrated by adding digitonin to a final concentration of 75 μg/ml and then adding 1 M EDTA at a 1:50 dilution and 10 N NaOH at a 1:700 dilution, and $[Ca^{2+}]_i$ was calculated as described (16).

Statistics. Data are reported as mean±SE. Statistical significance was assessed using an unpaired or paired Student's *t* test or ANOVA, as appropriate. For measurements of Na/H antiporter activity and $[Ca^{2+}]_i$, each data point represents a single measurement. For cAMP measurements, each data point represents the mean of three measurements.

Results

Binding of ET-1 to ET_B receptors activates NHE-3. In initial studies, 10^{-8} M ET-1 caused a 16% increase in Na/H antiporter activity in OKP cells (dpHi/dt = 0.94±0.04 pH U/min, control vs 1.09±0.04 pH U/min, ET-1; $n = 13$, control; $n = 11$, ET-1; $P < 0.01$). Whereas this result suggested that endothelin could activate NHE-3, this magnitude of regulation would likely be difficult to consistently reproduce and thus to study.

To examine whether increasing endothelin receptor abundance would increase the magnitude of this effect, we overexpressed ET_A and ET_B receptor cDNAs in OKP cells. As shown in Fig. 1 *A*, in pooled cells stably transfected with the neomycin resistance gene alone, 10^{-8} M ET-1 had no effect on antiporter activity. In three clones selected for highest expression of ET_A mRNA by Northern blot, ET-1 also failed to regulate Na/H antiporter activity. By contrast, in two clones selected for highest expression of ET_B mRNA, 10^{-8} M ET-1 increased Na/H antiporter activity significantly, by 26% in ET_B5 cells and by 39% in ET_B6 cells.

It is not clear why the pooled neomycin-resistant cells lost responsiveness to ET-1, but this was observed consistently. To examine this further, we measured ^{125}I-ET-1 binding in wild-type and ET_B6 cells. The results, shown in Table I, indicate that nonspecific binding was < 1% of specific binding in ET_B6 cells. In wild-type cells, no specific binding could be demon-

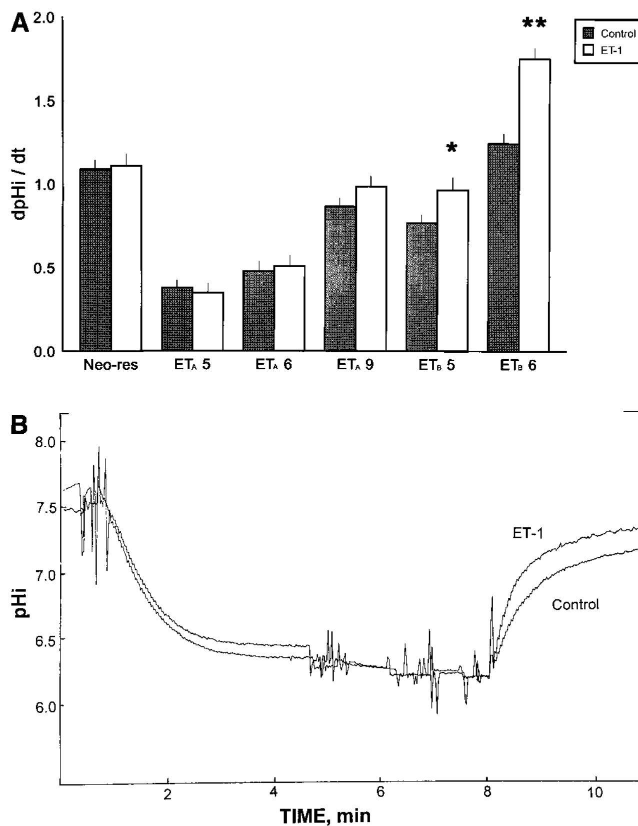

Figure 1. Na/H antiporter activity in OKP cells transfected with ET_A or ET_B receptor cDNAs. OKP cells were transfected with cDNAs for the ET_A or ET_B receptor, and clones were selected for highest expression by Northern blot. *Neo-res*, pooled cells transfected with pSV2-neo alone. (*A*) Na/H antiporter activity (pH U/min) is plotted in the absence and presence of 10^{-8} M ET-1. *$P < 0.01$ vs control; **$P <$ 0.001 vs control. Neo-res: control, $n = 6$, ET-1, $n = 7$; ET_A5: control, $n = 6$, ET-1, $n = 6$; ET_A6: control, $n = 10$, ET-1, $n = 10$; ET_A9: control, $n = 6$, ET-1, $n = 6$; ET_B5: control, $n = 6$, ET-1, $n = 6$; ET_B6: control, $n = 9$, ET-1, $n = 8$. (*B*) A typical tracing of Na/H antiporter activity in ET_B6 cells. Na^+ was added at 8 min.

strated. Thus, the small effect of ET-1 on Na/H antiporter activity in wild-type cells was mediated by a small unmeasurable number of receptors or was due to a promiscuous interaction with another receptor. It is possible that transfection or treatment with G-418 inhibited receptor activity.

Fig. 1 *B* shows a typical tracing of Na/H antiporter activity in ET_B6 cells. In ET_B6 cells, 10^{-10} M ET-1 increased Na/H antiporter activity by 10% ($P < 0.09$), and 10^{-9} M ET-1 increased Na/H antiporter activity by 32% ($P < 0.001$). All further studies were performed with 10^{-8} M ET- 1 in ET_B6 cells.

The above studies suggest that binding of ET-1 to ET_B receptors activates NHE-3. To confirm this, we examined the effect of receptor blockade in ET_B6 cells. Fig. 2 *A* shows results with 10^{-6} M FR-139137, an ET_A-selective antagonist, and 10^{-6} M PD145065, an antagonist of both ET_A and ET_B receptors (17). ET-1 induced activation of the Na/H antiporter was inhibited by the nonselective antagonist but not by the ET_A-selective antagonist, confirming an effect mediated by the ET_B receptor. Fig. 2 *B* shows that BQ788, a selective ET_B receptor blocker, completely inhibited ET-1–induced Na/H antiporter activation. Fig. 2 *C* shows that IRL-1620, a specific ET_B receptor agonist (18), increased Na/H antiporter activity by 46%. Thus, all of these studies demonstrate that the effect of ET-1 is mediated by the ET_B receptor.

In the above studies, Na/H antiporter activity was assayed 35 min after addition of ET-1. To determine the time course of the effect, 10^{-8} M ET-1 was added at varying times before initiation of the assay (nigericin addition). At 5 min, ET-1 increased Na/H antiporter activity by 11% (NS; $n = 7$, control; $n = 8$, ET-1); at 12 min, ET-1 increased Na/H antiporter activity by 48% ($P < 0.001$; $n = 7$); at 35 min, ET-1 increased Na/H antiporter activity by 40% ($P < 0.001$; $n = 8$). Thus, endothelin achieves a maximal effect within 12 min.

Role of adenylyl cyclase and protein kinase A. Protein kinase A is a potent inhibitor of the proximal tubule apical membrane Na/H antiporter. A number of hormones are believed to regulate Na/H antiporter activity through regulation of adenylyl cyclase and secondarily of protein kinase A activity (4). In ET_B6 cells, 10^{-8} M ET-1 inhibited PTH-stimulated cAMP production by 28% (Fig. 3). In the presence of PD145065, a nonselective endothelin receptor antagonist, 10^{-8} M ET-1 did not significantly affect cAMP production (115±11 vs 96±10 pmol/mg protein per h, control vs ET-1, NS, $n = 9$). In the presence of FR-139137, an ET_A- selective antagonist, 10^{-8} M ET-1 significantly inhibited cAMP production (117±11 vs 87±9 pmol/mg protein per h, $P < 0.05$, $n = 9$). Thus, binding of ET-1 to ET_B receptors inhibits adenylyl cyclase.

Table I. Binding of ^{125}I-ET-1

Concentration of cold ET-1, M	Counts: Wild-type cells	Counts: ET_B6 cells
0	121±14	13,932±875
10^{-10}	97±8	14,051±378
10^{-9}	100±2	8,482±585
10^{-8}	95±17	897±83
10^{-7}	103±6	68±10
10^{-6}	87±10	114±4

Mean±SD.

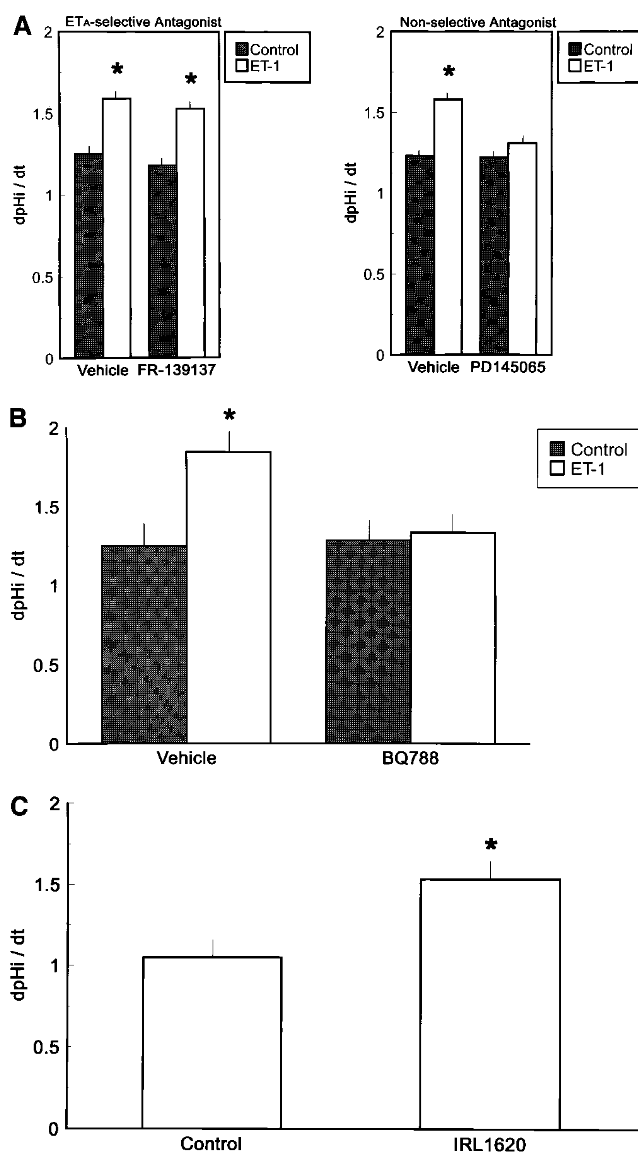

Figure 2. ET-1 increases Na/H antiporter activity via ET_B receptors. (*A*) Na/H antiporter activity (pH U/min) is plotted in the absence and presence of 10^{-8} M ET-1, and in the absence and presence of 10^{-6} M FR-139137, an ET_A-selective antagonist, or 10^{-6} M PD145065, a nonselective endothelin receptor antagonist. *$P < 0.001$ vs control. FR-139137: vehicle, control, $n = 10$, ET-1, $n = 10$; inhibitor, control, $n = 10$, ET-1, $n = 9$; PD145065: vehicle, control, $n = 10$, ET-1, $n = 10$; inhibitor, control, $n = 10$, ET-1, $n = 9$. (*B*) Na/H antiporter activity (pH U/min) is plotted in the absence and presence of 10^{-8} M ET-1, and in the absence and presence of 1.5×10^{-8} M BQ788, an ET_B-selective receptor antagonist. *$P < 0.05$ vs control. Vehicle, control, $n = 3$, ET-1, $n = 5$, Inhibitor, control, $n = 4$, ET-1, $n = 5$. (*C*) Na/H antiporter activity is plotted in the absence and presence of 10^{-8} M IRL-1620, a selective ET_B receptor agonist. *$P < 0.005$, $n = 7$.

To determine the role of inhibition of adenylyl cyclase and protein kinase A in NHE-3 regulation, three studies were performed. Fig. 4 shows results of studies examining the effect of 10^{-4} M 8-bromo cAMP on the response to ET-1. If ET-1 increases Na/H antiporter activity by inhibition of adenylyl cyclase, addition of excess exogenous cAMP should prevent this effect. ET-1 alone caused a 30% increase in Na/H antiporter

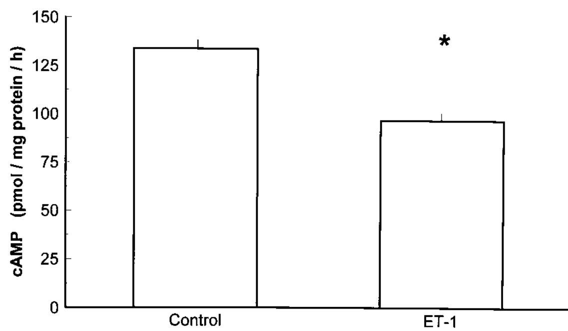

Figure 3. ET-1 inhibits PTH-stimulated cAMP production. cAMP production is plotted in the absence and presence of 10^{-8} M ET-1. *$P < 0.05$ vs control, $n = 12$.

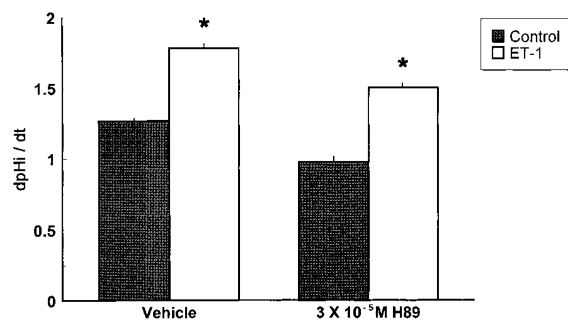

Figure 5. H89 does not inhibit ET-1–induced Na/H antiporter stimulation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was examined in ET_B6 cells in the absence and presence of 30 μM H89 (applied for 6 h). *$P < 0.002$ vs no ET-1, $n = 4$.

activity, and 8-bromo cAMP alone caused a 22% decrease in Na/H antiporter activity. In the presence of 8-bromo cAMP, ET-1 caused an increase in Na/H antiporter activity equal to 49% of the increase seen in the absence of 8-bromo cAMP. The observation that endothelin can activate the Na/H antiporter in the presence of exogenous cAMP demonstrates that regulatory mechanisms exist independently of adenylyl cyclase inhibition. The observation that the endothelin-induced increase in antiporter activity was smaller in magnitude in the presence of exogenous cAMP suggests that part of the stimulatory effect is mediated by inhibition of adenylyl cyclase. However, other explanations exist (see Discussion).

To address this further, we examined the effect of 30 μM H89, a protein kinase A inhibitor (19). As shown in Fig. 5, H89 did not inhibit ET-1–induced increases in Na/H antiporter activity. In the absence of H89, ET-1 increased Na/H antiporter activity by 0.52 pH U/min (41% increase), and in the presence of H89 ET-1 increased Na/H antiporter activity by 0.53 pH U/min (54% increase). To confirm that H89 inhibited regulation of the Na/H antiporter by protein kinase A in these cells, we examined the effect of H89 on forskolin-induced Na/H antiporter inhibition. 10^{-5} M forskolin inhibited Na/H antiporter activity by 30% ($n = 8$, $P < 0.005$). This effect was blocked by 5 μM H89 (forskolin caused a 7% inhibition; $n = 4$, NS), and by 10 μM H89 (forskolin caused an 11% increase; $n = 4$, NS).

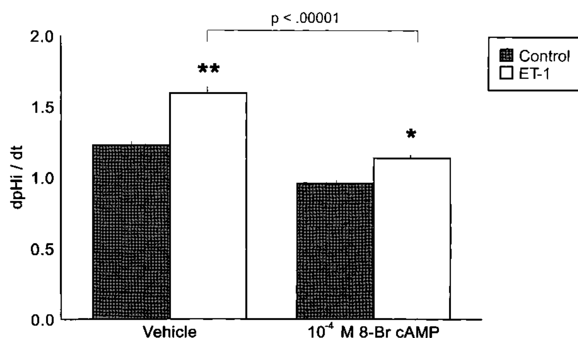

Figure 4. 8-Bromo cAMP partially inhibits ET-1 induced Na/H antiporter stimulation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was examined in ET_B6 cells in the absence and presence of 10^{-4} M 8-bromo cAMP. *$P < 0.05$ vs no ET-1; **$P < 0.001$ vs no ET-1. Control, $n = 14$; ET-1, $n = 15$; 8-Br cAMP, $n = 14$; 8-Br cAMP + ET-1, $n = 15$.

These results suggest that ET-1–induced inhibition of adenylyl cyclase does not contribute to the increase in Na/H antiporter activity. In agreement with this, H7, which inhibits protein kinase A and protein kinase C (19), did not inhibit endothelin-induced Na/H antiporter activation (see below).

To confirm these results further, we examined the effect of pertussis toxin pretreatment on ET-1–induced Na/H antiporter activation. Hormone-mediated adenylyl cyclase inhibition is frequently mediated by pertussis toxin–sensitive G proteins. Pretreatment of ET_B6 cells with 200 ng/ml pertussis toxin for 4 h inhibited ET-1–induced inhibition of cAMP accumulation. However, this maneuver did not inhibit ET-1–induced increases in Na/H antiporter activity. As shown in Fig. 6 *A*, 10^{-8} M ET-1 increased Na/H antiporter activity by 40% in vehicle-pretreated and in pertussis toxin–pretreated cells. Pertussis toxin pretreatment of cells ADP ribosylated all potential G protein substrates, as evidenced by the observation that pretreatment of cells inhibited subsequent in vitro pertussis toxin–induced ADP ribosylation of 41-kD proteins (Fig. 6 *B*). Once again, these results suggest that ET-1–induced increases in Na/H antiporter activity are not dependent on adenylyl cyclase inhibition.

Role of cell calcium and protein kinase C. Endothelin has been shown to activate phospholipase C, secondarily leading to increases in $[Ca^{2+}]_i$ and activation of protein kinase C (20). We therefore examined the role of these pathways in Na/H antiporter activation. As shown in Fig. 7 *A*, 10^{-8} M ET-1 caused a spike increase in $[Ca^{2+}]_i$, which was then followed by a smaller sustained increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increased from 106±9 nM to 263±14 nM at ~ 15 s and then decreased to a plateau of 133±9 nM ($n = 3$). Fig. 7 *B* shows the dose response curve for the peak increase in $[Ca^{2+}]_i$. A small effect was seen with 10^{-9} M, which increased in magnitude with 10^{-8} and 10^{-7} M ET-1.

The effect of ET-1 on $[Ca^{2+}]_i$ was mediated by the ET_B receptor. PD145065, the nonselective ET_A and ET_B receptor blocker, prevented the effect of 10^{-8} M ET-1 on $[Ca^{2+}]_i$, whereas FR-139137, the ET_A-selective antagonist, had no effect (Table II).

To further characterize the mechanism of the ET-1 induced increase in $[Ca^{2+}]_i$, a number of studies were performed (Table III). Pretreatment of cells with 200 ng/ml pertussis toxin for 4 h decreased and slowed but did not eliminate the peak Ca^{2+} response (Fig. 8 *A*, Table III). Removal of extracellular Ca^{2+} did not prevent the peak increase in $[Ca^{2+}]_i$ but prevented the sustained increase (Fig. 8 *B*, Table III). Treatment with exoge-

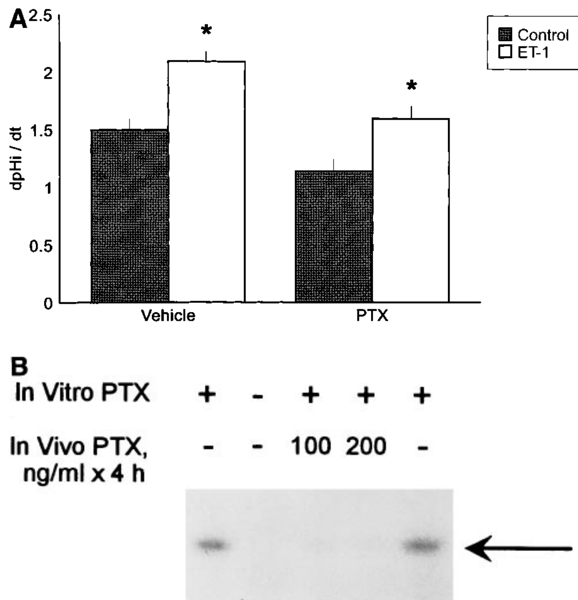

Figure 6. (*A*) Pretreatment of cells with pertussis toxin (*PTX*) does not inhibit ET-1–induced Na/H antiporter stimulation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was examined in ET_B6 cells pretreated with pertussis toxin (200 ng/ml for 4 h) or vehicle. $^{*}P < 0.001$ vs no ET-1. Control, $n = 7$; ET-1, $n = 8$; PTX, $n = 7$; PTX + ET-1, $n = 8$. (*B*) In vitro pertussis toxin–induced ADP ribosylation of membrane proteins. Cells were pretreated as indicated in the figure, and 100 μg of membrane protein subjected to in vitro ADP ribosylation in buffer containing (in mM): 50 Tris-HCl, pH 8.0, 10 thymidine, 2.5 $MgCl_2$, 1 ATP, 1 DTT, 0.5 EDTA, 0.01 NAD, 10 μCi ^{32}P-NAD, and 20 μg/ml activated toxin for 30 min at 30°C. Proteins were then precipitated in 40% TCA, resuspended in Laemmli sample buffer, and separated on 12.5% SDS-PAGE. The arrow denotes the 41-kD band.

nous 10^{-4} M 8-bromo cAMP and inhibition of tyrosine kinase pathways with 1 μM herbimycin A did not prevent the increase in $[Ca^{2+}]_i$. Thus, the increase in $[Ca^{2+}]_i$ is mediated by pertussis toxin sensitive and insensitive pathways, and involves release of Ca^{2+} from intracellular stores (peak) and Ca^{2+} entry (sustained increase). Tyrosine kinase– and cAMP-regulated pathways do not play a role.

To determine the role of increases in $[Ca^{2+}]_i$ in Na/H antiporter activation, increases in $[Ca^{2+}]_i$ were prevented by preincubating cells in 20 μM dimethyl BAPTA for 30 min before ET-1 addition. Whereas in control cells ET-1 increased $[Ca^{2+}]_i$ from 127±2 nM to 329±6 nM ($n = 3$), in BAPTA-treated cells ET-1 did not increase $[Ca^{2+}]_i$ (baseline $[Ca^{2+}]_i$ 98±1 nM; peak $[Ca^{2+}]_i$ 108±2 nM; $n = 5$). As shown in Fig. 9, prevention of $[Ca^{2+}]_i$ increases with BAPTA-inhibited ET-1–induced Na/H antiporter activation by 57%. In the absence of BAPTA, ET-1 increased Na/H antiporter activity by 49%, whereas, in the presence of BAPTA, ET-1 increased Na/H antiporter activity by only 21%. Thus, ET-1 binding to ET_B receptors increases $[Ca^{2+}]_i$, which mediates in part Na/H antiporter activation.

Increases in $[Ca^{2+}]_i$ can activate CaM–dependent kinase (CaM kinase). To determine if CaM kinase mediates Na/H antiporter activation, cells were treated with 40 μM KN62, an inhibitor of this kinase (19). In the absence of KN62, ET-1 increased Na/H antiporter activity by 39%, whereas, in the

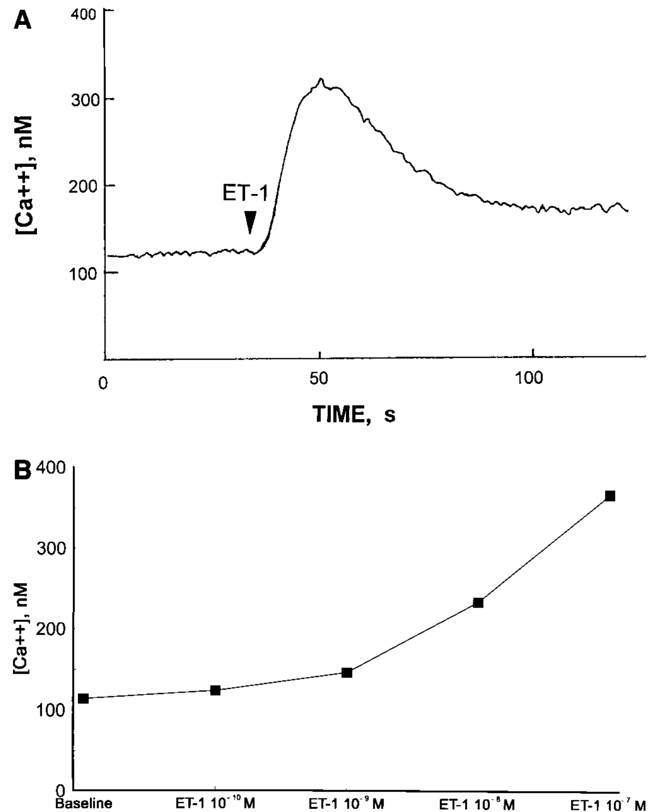

Figure 7. ET-1 increases $[Ca^{2+}]_i$. (*A*) A typical tracing showing the response of $[Ca^{2+}]_i$ to 10^{-8} M ET-1 in ET_B6 cells. (*B*) Peak $[Ca^{2+}]_i$ is plotted as a function of the concentration of ET-1; $n = 3$.

presence of KN62, ET-1 increased Na/H antiporter activity by only 19% (51% inhibition; Fig. 10).

Binding of ET to the ET_B receptor has been demonstrated to activate nitric oxide (NO) synthase, a process in which CaM kinase and tyrosine kinase pathways may play a role (21). To examine whether NO plays a role in ET-1–induced Na/H antiporter activation, cells were treated with 2 mM *N*-nitro-L-arginine (L-NA), an NO synthase inhibitor (22, 23). In the absence of L-NA, ET-1 increased Na/H antiporter activity by 34% (Fig. 11). L-NA caused a 46% decrease in baseline Na/H antiporter rate, but did not inhibit the effect of ET-1, which increased Na/H antiporter activity by 93% (Fig. 11).

Table II. ET-1-induced Increase in Cell Ca^{2+} Is Mediated by ET_B Receptor

Maneuver	$[Ca^{2+}]_i$ Baseline	$[Ca^{2+}]_i$ Peak	$[Ca^{2+}]_i$ Sustained	*n*
		(nM)		
1a. Control	122±9	301±41	150±14	4
1b. 10^{-6} M PD145065	108±6	114±10	111±8	4
2a. Control	108±2	237±3	135±5	4
2b. 10^{-6} M FR-139137	107±2	222±5	142±2	4

Mean±SE.

Figure 8. ET-1 increases $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is plotted as a function of time. (*A*) Effect of pertussis toxin. Cells were pretreated with 200 ng/ml pertussis toxin or vehicle for 4 h. (*B*) Effect of removal of extracellular Ca^{2+}. Studies were performed in the absence of extracellular Ca^{2+} with 2 mM EDTA.

Activation of phospholipase C can also lead to protein kinase C activation. To address whether protein kinase C plays a role in Na/H antiporter activation, cells were treated with 40 μM H7, an inhibitor of protein kinases A and C (19). This concentration of H7 completely inhibits phorbol ester–induced increases in c-*fos* mRNA abundance in OKP cells (24). However, H7 had no effect on ET-1–induced increases in Na/H antiporter activity (Fig. 12).

ET_A receptors have been found to increase cell $[Ca^{2+}]$ in many cell types (20). To examine whether increases in $[Ca^{2+}]_i$ were sufficient to increase Na/H antiporter activity, we examined $[Ca^{2+}]_i$ in ET_A6 cells. These cells had the highest levels of expression of ET_A mRNA but did not demonstrate an increase in Na/H antiporter activity in response to 10^{-8} M ET-1 (Fig. 1 *A*). ET-1 caused an increase in $[Ca^{2+}]_i$ in ET_A6 cells that was similar to that seen with ET_B6 cells (Table IV). ET-1 had no effect on $[Ca^{2+}]_i$ in pooled neomycin-resistant cells (Table IV). Thus, whereas increases in $[Ca^{2+}]_i$ are required for 50% of ET-1–induced Na/H antiporter stimulation, they are not sufficient.

Table III. Regulation of Cell Ca^{2+} Concentration

Maneuver	$[Ca^{2+}]_i$ Baseline	Peak	Sustained	*n*
		(nM)		
1. ----	106±9	263±14	133±9	3
2. 200 ng/ml Pertussis toxin	112±1	186±3	137±3	4
3. 0 extracellular Ca^{2+}	72±2	178±7	71±1	4
4. 10^{-4} M 8-Br cAMP	101±1	232±4	126±4	3
5. 1 μM HerbimycinA	130±4	381±8	147±5	5

Mean±SE.

Figure 9. BAPTA partially inhibits ET-1–induced Na/H antiporter stimulation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was compared in control ET_B6 cells and in cells preincubated with 20 μM dimethyl BAPTA for 30 min. $*P < 0.05$ vs no ET-1; $**P < 0.001$ vs no ET-1; $n = 7$.

Role of other signaling pathways. To examine if icosanoids play a role, the effects of indomethacin (cyclooxygenase inhibitor) and nordihydroguaiaretic (NDGA, a lipoxygenase and cytochrome P450 inhibitor) were examined (Table V). In the absence of indomethacin, ET-1 increased Na/H antiporter activity by 42%, whereas, in the presence of 20 μM indomethacin, ET-1 increased Na/H antiporter activity by 42%. In the absence of NDGA, ET-1 increased Na/H antiporter activity by 40%, whereas, in the presence of 10 μM NDGA, ET-1 increased Na/H antiporter activity by 48%.

Lastly, we examined whether protein kinase G played a role by examining the effect of 8-bromo cGMP. ET-1 increased Na/H antiporter activity by 43% ($P < 0.001$). 10^{-4} M 8-bromo cGMP had no effect on Na/H antiporter activity, and, in the presence of 8-bromo cGMP, ET-1 increased Na/H anti-

Figure 10. KN62 partially inhibits ET-1–induced Na/H antiporter stimulation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was compared in control ET_B6 cells and in cells pretreated with 40 μM KN62 for 30 min. $*P < 0.005$ vs no ET-1. Control, $n = 7$; ET-1, $n = 7$; KN62, $n = 8$; KN62 + ET-1, $n = 8$.

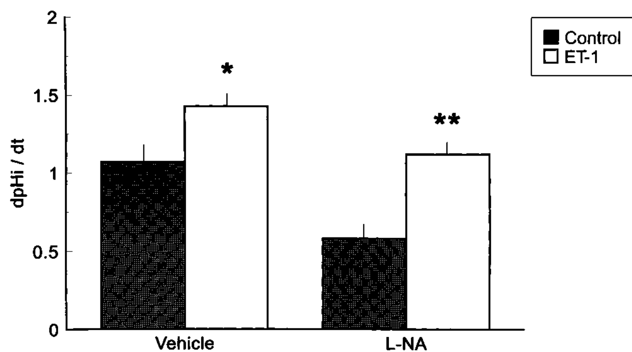

Figure 11. L-NA does not inhibit ET-1–induced Na/H antiporter activation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was compared in the absence and presence of 2 mM L-NA. *$P < 0.05$ vs no ET-1, **$P < 0.001$ vs no ET-1. Control, $n = 8$; ET-1, $n = 8$; L-NA, $n = 8$; L-NA + ET-1, $n = 9$.

porter activity by 35% ($P < 0.001$), an effect not different from that seen in the absence of 8-bromo cGMP.

Discussion

Previous studies have demonstrated that ET-1 increases Na/H antiporter activity in mesangial cells and Chinese hamster ovarian-K1 cells overexpressing endothelin receptors (25, 26). These cells express the ubiquitous Na/H antiporter isoform, NHE-1. The present studies demonstrate that ET-1 increases Na/H antiporter activity in OKP cells. This Na/H antiporter is encoded by the NHE-3 isoform (9), the isoform which encodes the apical membrane Na/H antiporter of the renal proximal tubule and thick ascending limb (5, 6). Overexpression of ET_B receptors significantly increased the response to endothelin, whereas overexpression of ET_A receptors was without effect. These results are not due to a failure of ET_A-transfected cells to express functional receptor, in that ET_A6 cells demonstrated an increase in $[Ca^{2+}]_i$ in response to ET-1. Pooled neomycin-resistant cells demonstrated no increase in $[Ca^{2+}]_i$. A role for the ET_B receptor was confirmed by the demonstration that ET-1–induced increases in Na/H antiporter activity were not blocked by an ET_A-selective endothelin receptor antagonist but were blocked by a nonselective ET_A and ET_B receptor antagonist and an ET_B-selective receptor antagonist. In addition, IRL-1620, a selective ET_B receptor agonist, increased Na/H antiporter activity in these cells.

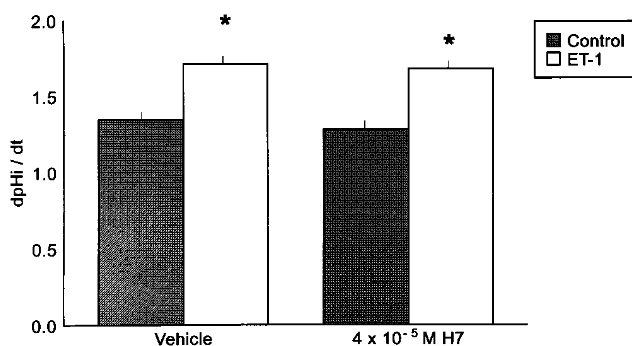

Figure 12. H7 does not inhibit ET-1–induced Na/H antiporter stimulation. The effect of 10^{-8} M ET-1 on Na/H antiporter activity (pH U/min) was examined in ET_B6 cells in the absence and presence of 40 μM H7. *$P < 0.001$ vs no ET-1. Control, $n = 9$; ET-1, $n = 9$; H7, $n = 8$; H7 + ET-1, $n = 9$.

Table IV. ET_A Receptor Increases Cell Ca^{2+}

Cells	$[Ca^{2+}]_i$ Baseline	Peak	Sustained	*n*
		(nM)		
1. ET_A6 cells, 10^{-8} M ET-1	67±5	221±7	106±5	3
2. ET_A6 cells, 10^{-7} M ET-1	73±4	297±6	96±4	3
3. Neomycin resistant, 10^{-8} M ET-1	121±3	122±4	122±4	4
4. Neomycin resistant, 10^{-7} M ET-1	136±8	138±7	138±7	3

Mean±SE.

Investigators have been unable to demonstrate significant expression of ET_A or ET_B receptor mRNA in the proximal tubule using reverse transcriptase–PCR (27). However, endothelin binds to S1 proximal tubule cells, with ET-1 and ET-3 binding of similar magnitude, consistent with the presence of ET_B receptors (28). Whereas ET-1 and ET-2 interact with ET_A and ET_B receptors, ET-3 interacts with high affinity only with ET_B receptors (1, 2). Thus, ET-3 can serve as a selective activator of ET_B receptors. Parallel roles for ET-3 and ET_B receptors are suggested by the observation that genetic disruption of the ET-3 and ET_B genes leads to similar phenotypes (29, 30). The proximal tubule expresses significant amounts of ET-3 mRNA, raising the possibility that ET-3 could function as an autocrine factor (31). ET-1 is also expressed in the proximal tubule, but in lesser amounts (32).

In ET_B6 cells, ET-1 caused a spike increase followed by a sustained increase in $[Ca^{2+}]_i$. This finding agrees with previous results in many cell types, which have found that ET_A and ET_B receptors activate phospholipase C, leading to increases in inositol-1,4,5-trisphosphate and $[Ca^{2+}]_i$ (20, 33). The mechanism of the increase in $[Ca^{2+}]_i$ is similar to that seen with many G protein–coupled receptors. Ligand binding to receptor activates phospholipase C through pertussis toxin–sensitive and –insensitive G protein pathways. The pertussis toxin–insensitive G protein is likely $G_{\alpha q}$ or a related G protein ($G_{\alpha 11}$, $G_{\alpha 14}$, and Gα16) (34). Pertussis toxin–sensitive increases in $[Ca^{2+}]_i$ may be due to $G_{\alpha i}$ or $G_{\beta\gamma}$ (34). Phospholipase C is then activated, leading to increases in inositol-1,4,5-trisphosphate release with secondary release of Ca^{2+} from intracellular stores. This then results in an opening of plasma membrane Ca^{2+} channels, repleting intracellular Ca^{2+} stores and maintaining the sustained increase in $[Ca^{2+}]_i$.

The role played by Ca^{2+}-dependent pathways in Na/H antiporter activation is complex. BAPTA prevented the ET-1–induced increase in $[Ca^{2+}]_i$ and inhibited approximately half of the ET-1–induced increase in Na/H antiporter activity. Similarly, KN62, an inhibitor of CaM kinase, inhibited 50% of ET-1–induced antiporter activation. These results suggest that increases in $[Ca^{2+}]_i$ and secondary activation of CaM kinase mediate 50% of ET-1–induced Na/H antiporter activation. Whereas increases in $[Ca^{2+}]_i$ are required for this component of antiporter stimulation, they are not sufficient. In ET_A6 cells, 10^{-8} M ET-1 increased $[Ca^{2+}]_i$ but did not increase Na/H antiporter activity. In the proximal tubule, increases in $[Ca^{2+}]_i$ have been proposed to stimulate and inhibit Na and H trans-

Table V. Effect of Indomethacin and NDGA on ET-1–induced Activation of the Na/H Antiporter

	Vehicle			Inhibitor		
	dpH_i/dt, pH U/min			dpH_i/dt, pH U/min		
	Control	ET-1	% increase	Control	ET-1	% increase
Indomethacin, 20 μM	0.76±0.07	1.08±0.05	42%**	0.69±0.03	0.98±0.08	42%*
NDGA, 10 μM	1.34±0.13	1.88±0.03	40%**	1.04±0.09	1.54±0.15	48%**

$^*P < 0.001$, $^{**}P < 0.005$, Mean±SE.

port (35–37). Studies in renal cortical apical membranes have suggested that CaM kinase directly inhibits the Na/H antiporter (38). Thus, the effect of increases in $[Ca^{2+}]_i$ and CaM kinase on the Na/H antiporter are likely complex, depending on many possible variables, including activation of parallel pathways, subcellular localization of Ca^{2+}, and other yet to be defined factors.

Although phospholipase C activation would also be expected to increase diacylglycerol levels and activate protein kinase C, inhibition of protein kinase C with H7 had no effect on activation of the Na/H antiporter by ET-1. This result is different from that of Walter et al., who found that calphostin C inhibited ET-1–induced increases in Na/H antiporter activity in OK cells (39). The reason for this discrepancy is not clear, but it may relate to differences in the cells used. The present studies also found no role for protein kinase G, cyclooxygenase, lipoxygenase, or cytochrome P450 pathways.

The present studies confirm that ET-1 binding to ET_B receptors causes inhibition of adenylyl cyclase activity, as found previously (8, 40). Also in agreement, addition of ET-1 to ET_B overexpressing Chinese hamster ovarian cells inhibits adenylyl cyclase, whereas addition to ET_A-overexpressing cells stimulates adenylyl cyclase (33). However, regulation of adenylyl cyclase likely does not mediate regulation of NHE-3, in that H89 and H7, inhibitors of protein kinase A (19), did not inhibit the endothelin-induced increase in Na/H antiporter activity. To prove that H89 inhibited protein kinase A in these cells, we showed that H89 blocked forskolin-induced Na/H antiporter inhibition. Lastly, we showed that pertussis toxin pretreatment, which inhibits ET-1–induced inhibition of adenylyl cyclase, did not inhibit Na/H antiporter activation. The observation that exogenous 8-bromo cAMP addition partially inhibited endothelin-induced Na/H antiporter activation may be attributable to regulation of an endothelin-signaling pathway by protein kinase A, or may be due to the fact that protein kinase A is a potent inhibitor of Na/H antiporter activity and thus prevents activation by other agonists. We have previously found that exogenous 8-bromo cAMP inhibited angiotensin II–induced Na/H antiporter stimulation in OKP cells, in spite of the fact that angiotensin II had no effect on cAMP production in these cells (41).

In the in vitro–perfused proximal straight tubule, low concentrations of endothelin stimulate volume absorption whereas high concentrations inhibit volume absorption (3). ET-1 has been demonstrated to increase proximal tubule apical membrane Na/H antiporter activity (7, 8). The present studies demonstrate that ET binding to ET_B receptors leads to an increase in NHE-3 activity. Thus, stimuli that increase renal production of ET-1 or ET-3 could secondarily increase Na/H antiporter activity, which would lead to an increase in renal sodium reabsorption. Endothelin-induced increases in $[Ca^{2+}]_i$ mediate 50% of the increase in Na/H antiporter activity. In recent studies we have found that herbimycin A and tyrphostin A47, tyrosine kinase inhibitors, also prevent 50% of ET-1–induced Na/H antiporter activation (unpublished observation). Herbimycin A and BAPTA together completely prevent antiporter activation. In the present studies we found that herbimycin A does not inhibit ET-1–induced increases in cell $[Ca^{2+}]$. Thus, Ca^{2+}- dependent and tyrosine kinase pathways function in parallel, and each mediates 50% of endothelin-induced Na/H antiporter stimulation.

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