Endothelin Inhibits Vasopressin-stimulated Water Permeability in Rat Terminal Inner Medullary Collecting Duct

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Abstract

Renal tubule solute and water transport is subject to regulation by numerous factors. To characterize direct effects of the recently discovered peptide endothelin (ET) on renal tubule transport, we determined signaling mechanisms for ET effects on vasopressin (AVP)-stimulated water permeability (P_F) in rat terminal inner medullary collecting duct (IMCD) perfused in vitro. ET caused a rapid, dose-dependent, and reversible fall in AVP- but not cyclic AMP–stimulated P_F, suggesting that its effect on P_F is by inhibition of cyclic AMP accumulation. Indomethacin did not block ET actions, ruling out a role for prostaglandins in its effect. The protein kinase C (PKC) inhibitor calphostin, or pretreatment of perfused tubules with pertussis toxin, blocked ET-mediated inhibition of AVP-stimulated P_F. ET caused a transient increase in intracellular calcium ($[Ca^{2+}]_i$) in perfused tubules, an effect unchanged in zero calcium bath or by PT pretreatment. ET effects on P_F and $[Ca^{2+}]_i$ desensitized rapidly. Inhibition of P_F was transient and largely abolished by 20 min ET preexposure, and repeat exposure to ET did not alter $[Ca^{2+}]_i$. In contrast, PGE_2-mediated inhibition of AVP-stimulated P_F and increase of $[Ca^{2+}]_i$ were sustained and unaltered by prior exposure of IMCD to ET. Thus desensitization to ET is homologous. We conclude that ET is a potent inhibitor of AVP-stimulated water permeability in rat terminal IMCD. Signaling pathways for its effects involve both an inhibitory guanine nucleotide–binding protein and phospholipase-mediated activation of PKC. Since ET is synthesized by IMCD cells, this peptide may be an important autocrine modulator of renal epithelial transport. (*J. Clin. Invest.* 1992. 90:1458–1466.) Key words: cyclic AMP • protein kinase C • G protein • desensitization • indomethacin

Introduction

Renal epithelial transport of solute and water is subject to regulation by a variety of factors. The recently discovered peptide endothelin (ET)[1] (1) has been shown to modulate numerous renal functions, mediated by various cellular signaling mechanisms (2). ET increases renal vascular resistance and contracts glomerular mesangial cells, resulting in decreases in renal blood flow and glomerular filtration rate (2–4). It also results in augmented natriuresis and diuresis when infused at subpressor doses (3, 5). These latter effects suggest that ET could have direct effects on renal tubule transepithelial transport processes. Evidence consistent with this possibility derives from studies that demonstrate inhibition by ET of Na/K-ATPase in rabbit medullary collecting ducts (6), inhibition of vasopressin (AVP)-stimulated cyclic AMP accumulation in rat inner medullary collecting duct (IMCD) (7), and inhibition of luminal sodium entry in rabbit cortical collecting duct (8). The demonstration that rat IMCD cells in culture synthesize ET (9) suggests that ET could have an important autocrine function in regulation of transport.

Despite abundant evidence for modulation by ET of renal epithelial cell processes involved in transepithelial transport, direct regulation of such transport has been demonstrated only by Oishi et al. (10), who showed that ET inhibits AVP-stimulated water permeability, but not urea permeability, in rat IMCD. The present studies were done to characterize further the direct effects of ET on renal transepithelial transport by determining signaling mechanisms involved in its effects on water permeability (P_F) in rat terminal IMCD. Our results demonstrate potent direct inhibition of AVP-stimulated P_F by ET in this segment, which involves multiple signaling processes and, at least in vitro, desensitizes rapidly.

Methods

Tissue preparation. Tubules were perfused in vitro according to general methods originally described by Burg et al. (11) with modifications (12–14). Briefly, barrier-raised pathogen-free male Sprague-Dawley rats (Charles River, Montreal, Quebec) weighing 75–120 g were killed by decapitation and the kidneys were rapidly removed. Rats had free access to food (sodium content 196 meq/kg) and water and no diuresis was induced before death. Coronal slices including the papillary tip were cut and placed in chilled bathing solution previously equilibrated at 37°C with 95% air/5% CO_2. IMCD segments were dissected from along the inner medullary axis, care being taken to ensure that the proximal end of the dissected tubule originated distal to the end of the proximal third of the inner medulla. The tubule was transferred to a thermostatically controlled perfusion chamber containing bathing solution and mounted on pipettes as described previously (12, 13). Perfused tubule length averaged 790±30 μm. The temperature of the bath was slowly increased to and maintained at 37°C. The bathing solution contained the following (mM): 215 Na, 203 Cl, 25 HCO_3, 5 K, 10 urea, 1.5 Ca, 1.2 Mg, 5 NH_4, 8 glucose, 5 Hepes, and 1 g/liter albumin, and was equilibrated with 5% CO_2/95% air at 37°C before each experiment. The pH was 7.4 and osmolality averaged 442 mosmol/kg H_2O. Bath solution was loaded into 60 cm^3 syringes and pumped continuously via polyethylene tubing onto the bottom of one end of the perfusion chamber, while bath was continuously suctioned off the top of the other end of the perfusion chamber through polyethylene tubing attached to continuous vacuum.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular calcium; ET, endothelin; IMCD, inner medullary collecting duct; IP_3, inositol triphosphate; 8-*p*-CPT-cAMP, 8-para-chlorophenylthio-cAMP; P_F, water permeability; PKC, protein kinase C.

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Tubules were initially perfused with albumin-free bathing solution. Approximately 15–20 min after the initiation of perfusion, the perfusate was changed to an isotonic solution (osmolality 305 mosmol/kg) identical to the hypertonic perfusate but containing 75 mM less NaCl. The perfusate also contained exhaustively dialyzed [^{3}H]inulin as a volume marker. Water reabsorption was measured by making timed collections of perfused fluid into a constant-volume constriction pipette.

Measurement of intracellular calcium [Ca^{2+}]$_i$. In experiments in which [Ca^{2+}]$_i$ was measured, the perfusion chamber was made smaller to allow for rapid complete bath fluid exchange. Tubules were dissected and connected to perfusion pipettes as described above. The perfusate was the same as hypertonic perfusate but was Ca^{2+}-free and contained 50 μM EGTA. Tubules were loaded for 45 min at 31°C with 1.25 μM of the acetoxy-methyl ester of fura-2 (fura-2/AM) in standard bathing solution. Fura-2 was then washed away, bath temperature was increased to 37°C, and bath exchange rate was increased to 2 ml/min. Intracellular fura-2 fluorescence intensity at 520 nm was measured by photon counting with continuous rapidly alternating excitation from dual monochromators set at 340 and 380 nm (Deltascan; Photon Technology International, New Brunswick, NJ). The monochromator output was coupled to the inverted microscope through a 400-nm dichroic mirror and an oil immersion 100× fluor objective. Background and autofluorescence counts, determined before loading of the tubule with fura-2, were < 10% of fura-2–loaded values and were subtracted from all measurements. The ratio of emission intensity at 340 and 380 nm (340:380 ratio, R) was continuously monitored.

In situ calibration of [Ca^{2+}]$_i$ was performed at the end of each experiment. The bath was changed to a nominally Ca^{2+}-free solution containing 2 mM EGTA and 16.7 μM of the calcium ionophore 4Br-A23187. After determination of a stable 340:380 ratio (R_{min}), tubules were exposed to standard bath solution containing the same concentration of 4Br-A23187, and the 340:380 ratio (R_{max}) was determined again.

Calculations. The osmotic water permeability coefficient P_F (μm/s) was determined from $P_F = RTL_p/V_w$, where V_w is the partial molal volume of water, R and T have their usual meanings, and L_p, the hydraulic conductivity, was determined according to DuBois et al. (15): $L_p = 1/RTSC_B^2\{C_B(V_0 - V_1) + C_0V_0\ln[(C_B - C_0)V_0/(C_BV_1 - C_0V_0)]\}$. V_1 is the perfused fluid collection rate (nl/min), measured directly, and V_0 is the initial perfusion rate (nl/min), calculated from $V_0 = V_1(C_1^*/C_0^*)$, where C_1^* and C_0^* represent dpm/nl of [^{3}H]inulin in collected fluid and perfusate, respectively. S is the luminal surface area calculated from lumen diameter (assuming the tubule to be a perfect cylinder) and tubule length, measured directly by eyepiece micrometer at the end of each experiment. C_0 and C_B represent perfusate and bath osmolalities, respectively. This calculation of P_F assumes that no net solute flux occurs and, therefore, changes in luminal osmolality result only from water reabsorption. Sands et al. (16, 17) have shown that this assumption is valid in microperfused IMCD. This calculation also assumes that an effective osmotic gradient exists along the entire length of the perfused tubule. To ensure that this was true, tubules were perfused at > 20 nl/min. These high perfusion rates also minimize any effect of small net solute fluxes (18, 19) on luminal tonicity.

[Ca^{2+}]$_i$ was calculated as $K_d[(R - R_{min})/(R_{max} - R)][380_{min}/380_{max}]$, where K_d is the dissociation constant for fura-2–Ca^{2+}, assumed to be 224 nM at 37°C, and 380_{min} and 380_{max} represent fluorescent emission intensity at 380 nm excitation when R_{min} and R_{max} were determined (20).

Protocols. Because tubule-to-tubule variability often occurs in hormone responsiveness, experiments were designed to make paired comparisons of ET effects in each tubule. Effects of ET on AVP-stimulated P_F were studied under four conditions: AVP alone, AVP in the presence of indomethacin, AVP in the presence of calphostin, and AVP after pertussis toxin pretreatment of perfused tubules. In general, after the change to isotonic perfusate containing [^{3}H]inulin, 15–30 min elapsed before the addition of AVP to the bath. In some tubules, two to three collections were made before AVP addition to determine basal (AVP-independent) P_F. AVP alone was present in the bath for 45–50 min (control period), after which ET was added (experimental period). This was accomplished by exchanging the bath syringe for one containing both ET and AVP. Tubules were exposed to ET for 30–35 min, after which ET was removed from the bath (recovery period). In some experiments, the experimental period lasted ~ 60 min. Perfused fluid collections ranging from 6 to 8 min in duration were made continuously, beginning 20–25 min after addition of AVP to the bath.

In all experiments, the concentration of AVP used was 5 pM. In preliminary experiments, this concentration of AVP resulted in a stable P_F value, which averaged 89±7% (n = 5) of that obtained with exposure of tubules to 50 pM AVP and 90±3% (n = 4) of that obtained after exposure to 100 pM AVP. Thus 5 pM AVP results in a close to maximal permeability response, allowing for increased sensitivity in detection of inhibitory effects of other agents.

To determine whether ET antagonism of AVP-stimulated P_F in rat IMCD occurred at a site before or subsequent to the generation of cyclic AMP, a series of experiments were done in which P_F was stimulated with the nonhydrolyzable cyclic AMP analogue 8-para-chlorophenylthio-cAMP (8-p-CPT-cAMP). Experiments with the cyclic AMP analogue were carried out using an identical protocol, with AVP replaced by the analogue.

In experiments designed to determine the PG dependence of ET action, indomethacin (10 μM) was present in dissecting solution and in all bathing solutions.

In experiments designed to determine the protein kinase C (PKC) dependence of ET action, we used the microbial product calphostin, a recently discovered PKC inhibitor with much greater specificity than other known PKC inhibitors (21). Calphostin (10^{-7} M) was added to the bath at the same time as AVP and remained in the bath throughout the experiment. This concentration of calphostin is close to the IC_{50} value for PKC inhibition but well below that for other protein kinases (21). All experiments were done under standard fluorescent room lighting conditions (22).

In experiments designed to determine whether ET signals via a pertussis toxin–sensitive pathway, pertussis toxin (500 ng/ml) was added to bath solution after initiation of tubule perfusion, at 37°C, before addition of AVP to the bath. After a 1-h exposure, the pertussis toxin was washed away, and collections to determine AVP-independent P_F were made before addition of AVP to the bath. The remainder of the protocol was identical to that described above.

In experiments designed to assess desensitization to effects of ET, a second exposure to either ET or PGE_2 occurred 30–35 min after removal of ET from the bath, always with continuous exposure to AVP.

Reagents. ET-1 was used in all experiments and was purchased from Peninsula Laboratories (Belmont, CA). AVP, 8-p-CPT-cAMP, indomethacin, PGE_2, and pertussis toxin were purchased from Sigma Chemical Co. (St. Louis, MO), calphostin was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA), fura-2/AM, and 4Br-A23187 were purchased from Molecular Probes Inc. (Eugene, OR), and [^{3}H]methoxy-inulin was purchased from New England Nuclear (Boston, MA).

Statistics. For statistical comparisons, a single value of P_F was determined for each experimental period by averaging values obtained generally from the two to three collections at the end of each period. However, as shown in the results section and in Fig. 1, the inhibitory effect of ET on AVP-stimulated P_F was transient. Therefore, the P_F values obtained in the second and third collections after addition of ET to the bath, when the inhibitory effect was maximal, were averaged to obtain a single P_F value for that period.

For each experiment in which [Ca^{2+}]$_i$ was measured, a mean value for steady state [Ca^{2+}]$_i$ was determined by integration of the concentration-time curve over a 30–60 s time period before the addition of ET to the bath. This value was then compared with the peak [Ca^{2+}]$_i$ achieved after ET was added to the bath.

The value n represents the number of tubules for each protocol. Data are presented as means ± SE, and statistical comparisons were made by use of the two-tailed paired t test. Intergroup comparisons

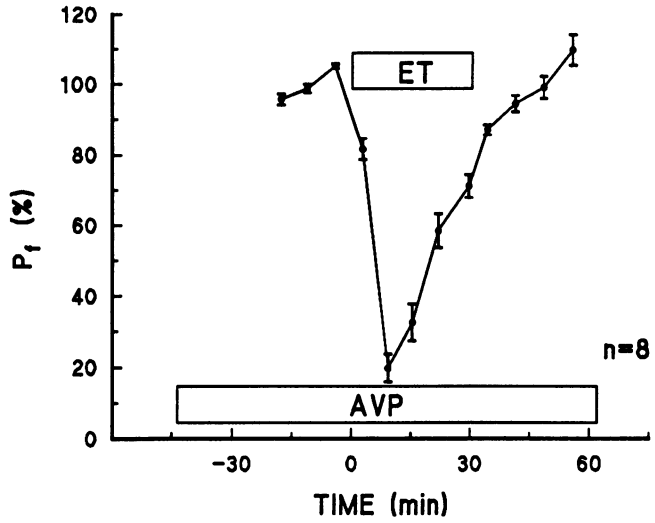

Figure 1. ET (10 nM) effect on AVP (5 pM)-stimulated P_F. Mean P_F±SE, expressed as percentage of mean control period (pre-ET) P_F is plotted on *y*-axis. Midpoint (mean) of each collection in relation to time of addition of ET is plotted on *x*-axis. Boxes indicate time and duration of exposure to indicated agents. See text for details and statistical analysis.

were made using the unpaired *t* test. Where multiple groups were involved, one-way analysis of variance (ANOVA) was used, followed by the least squares method to compare groups.

Results

Endothelin effects on AVP-stimulated P_F

Initial experiments were designed to document the effects of endothelin on AVP-stimulated P_F. Results are depicted in Fig. 1 and Table I. AVP-stimulated P_F averaged 980±71 μm/s and decreased rapidly to 244±42 μm/s ($P < 0.001$) after addition of ET (10 nM) to the bath. This effect was reversible, as removal of ET from the bath resulted in return of P_F to values not different than pre-ET values (Table I). As shown in Fig. 1, the inhibition of P_F by ET was invariably detectable in the first collection after initiating the bath solution change. The midpoint of this collection was ~ 3 min after initiation of the bath solution change. Maximal inhibition was noted in the second collection, after which P_F increased towards pre-ET values. Since the bath solution change took ≤ 2–3 min to complete, and a small yet significant dead space volume exists in the perfused fluid collection pipette, it was not possible to define the time course of the change in P_F more precisely. Nevertheless, the time course of the inhibitory effect of ET is distinctly different than that which occurs with PGE_2 inhibition of AVP-stimulated P_F in this segment (14) (see Fig. 6). In experiments in which ET was left in the bath for up to 60 min, P_F returned to pre-ET values in continued presence of ET.

In four tubules, the effect of 1 nM ET was determined. This concentration of ET resulted in a 40±9% fall in P_F. Thus, dose-dependent effects of ET occur over a concentration range close to the K_d for ET binding to papillary membranes (23).

In separate experiments ($n = 5$), the effect of ET on P_F in the absence of AVP was determined. P_F averaged 20±7 μm/s under basal conditions, 8±7 μm/s in the presence of 10 nM ET ($P > 0.2$), and 8±3 μm/s after removal of ET from the bath. Thus, ET alone has no effect on P_F.

ET effects on cyclic AMP-stimulated P_F

Antagonism of AVP-stimulated P_F may occur at sites beyond the generation of cAMP (14, 24). To determine whether this is also true for ET antagonism of P_F in rat IMCD, P_F was stimulated with the nonhydrolyzable cyclic AMP analogue 8-*p*-CPT-cAMP before addition of ET to the bath. The results are depicted in Fig. 2 and Table I. In contrast to the ET–AVP interaction, there was no detectable inhibitory effect of ET (10 nM) on cAMP-stimulated P_F.

Mechanism of ET inhibition of AVP-stimulated P_F

Role of endogenous PGs. Studies exist suggesting an important role for intrarenally produced PGs as mediators of ET effects in the kidney, including effects in medullary collecting ducts (2, 6). To determine whether this might also be true for ET inhibition of AVP-dependent P_F, the protocol with AVP and ET was repeated in the presence of the cyclooxygenase inhibitor indomethacin, added to the bath at a concentration of 10 μM (7, 25, 26). The results are summarized in Table I. AVP-stimulated P_F averaged 1,218±113 and fell to 497±157 μm/s with addition of ET to the bath.

Role of PKC. In most tissues examined, ET results in the generation of inositol trisphosphate (IP_3) (27) and diacyl glyc-

Table I. Summary of Microperfusion Experiments

Agonist	Antagonist-1	Antagonist-2	Bath additions	*n*	P_F Agonist alone	P_F Agonist plus antagonist-1	P_F Recovery period	P_F Agonist plus antagonist-2
AVP	ET	—	—	8	980±71	244±42*	1,021±77	
AVP	ET	ET	—	6	1,131±118	312±116‡	1,055±133	1,038±60
AVP	ET	PGE_2	—	4	922±132	316±53§	878±82	325±56¶
8-CPT-cAMP	ET	—	—	5	571±120	578±137	637±174	
AVP	ET	—	Indomethacin	4	1,218±113	497±157‖	1,279±96	
AVP	ET	—	Calphostin	5	681±71	633±70	610±61	
AVP	ET	—	Pertussis toxin (pre)	4	1,573±169	1,513±207	1,797±219	

P_F, water permeability coefficient (μm/s); AVP, vasopressin, 5 pM; ET, endothelin, 10 nM; PGE_2, 0.1 μM; Indomethacin, 10 μM; calphostin, 0.1 μM; pertussis toxin, 500 ng/ml in bath for 1 h before addition of AVP. See text for details of protocols. * $P < 0.001$ versus agonist alone; ‡ $P < 0.01$ versus agonist alone; § $P < 0.02$ versus agonist alone; ‖ $P < 0.05$ versus agonist alone; ¶ $P < 0.02$ versus recovery period.

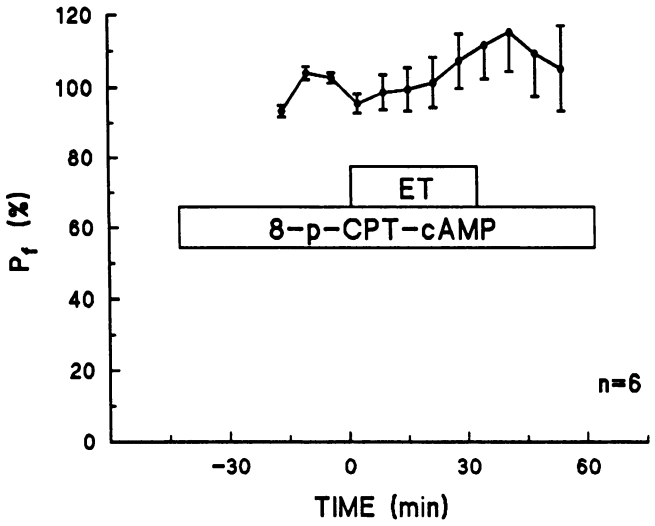

Figure 2. ET (10 nM) effect on 8-*p*-CPT-cAMP (0.1 mM)-stimulated P_F. See text and legend to Fig. 1 for details.

erol, with consequent activation of PKC (28, 29). Since agonist-stimulated PKC activation has been shown to mediate inhibition of AVP-stimulated P_F (30) and is involved in ET inhibition of cAMP accumulation in IMCD (7), we determined whether PKC activation might be involved in the functional effect of ET in IMCD. The above protocol was repeated in the presence of calphostin. Results of these experiments are depicted in Fig. 3 and Table I. In the presence of 0.1 μM calphostin, AVP-stimulated P_F averaged 681±71 μm/s. Subsequent addition of ET to the bath had no significant effect on P_F, which averaged 633±70 μm/s. Thus, inhibition of PKC activity blocks ET-mediated inhibition of AVP-stimulated P_F, consistent with a role for PKC in mediating functional effects of ET in IMCD. In the presence of calphostin, AVP-stimulated P_F was significantly lower ($P < 0.05$) than in tubules exposed to AVP alone ($P < 0.01$ for ANOVA on four AVP groups). In separate paired experiments ($n = 4$), we determined the effect of calphostin on the established AVP-stimulated P_F. AVP-stimulated P_F averaged 1,160±111 μm/s. 30–55 min after addition of calphostin to the bath, P_F averaged 1,057±147 μm/s or 90±5% of control values (P = NS). Since the calphostin effect is irreversible (22), no recovery period was performed for these experiments.

Role of pertussis toxin-sensitive G proteins. Since inhibition of AVP-stimulated cAMP production by antagonists may be mediated via guanine nucleotide regulatory proteins sensitive to inactivation by pertussis toxin (e.g., G_i), we determined whether such a signaling pathway is involved in ET-mediated inhibition of P_F. Results are shown in Table I. After exposure of perfused tubules to pertussis toxin, but before addition of AVP to the bath, P_F averaged 8±4 μ/s (n = 4), a value not different than the mean AVP-independent P_F of 22±5 μm/s (n = 14) observed in other tubules. Addition of AVP to pertussis toxin–treated tubules resulted in an increase of P_F to 1,573±169 μm/s, a value significantly greater than that observed in the group of tubules exposed to AVP alone ($P < 0.05$ after ANOVA). After addition of ET to the bath, P_F did not change, averaging 1,513±207 μm/s ($P > 0.5$). Thus, ET inhibition of AVP-stimulated P_F likely involves signaling through a pertussis toxin–sensitive G protein.

Desensitization of ET effect on AVP-stimulated P_F

As described above and as shown in Fig. 1, the inhibitory effect of ET on AVP-stimulated P_F desensitized rapidly. To determine the duration and possible mechanism of this desensitization, we carried out prolonged protocols in which tubules stimulated by AVP were exposed to ET twice. Results are depicted in Fig. 4 and Table I. The initial exposure to ET resulted in the same pattern of inhibition of P_F as described above (Fig. 1). P_F returned to control (AVP alone) values after removal of ET from the bath. The repeated addition of ET to the bath, however, was without effect on P_F. Thus the desensitization to this effect of ET is prolonged (≥30 min).

To ensure that tubule cell processes necessary for inhibition of P_F were still functional during the second exposure to ET, we used PGE_2, which we have previously shown to cause a rapid

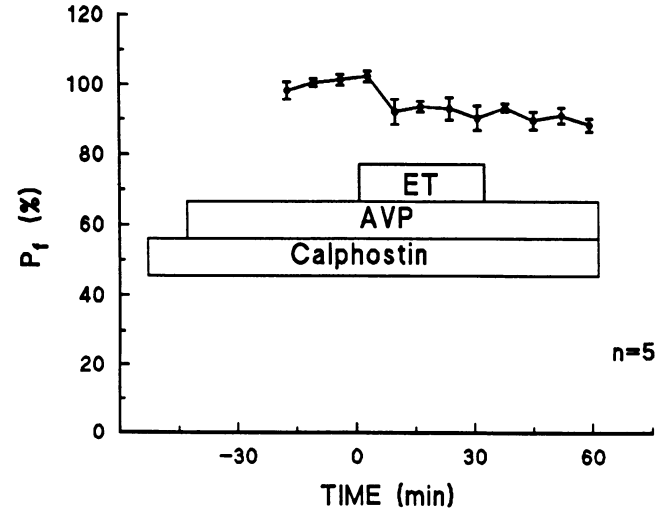

Figure 3. Effect of PKC inhibition with calphostin (0.1 μM) on ET (10 nM)-mediated inhibition of AVP (5 pM)-stimulated P_F. See text and legend to Fig. 1 for details.

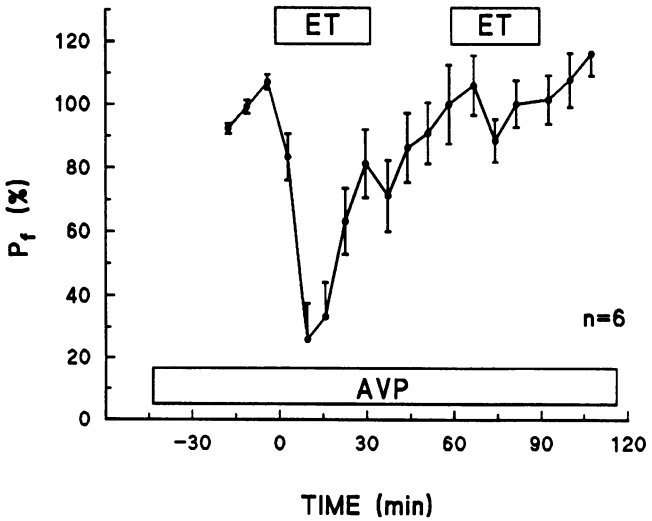

Figure 4. Desensitization of ET effect. See text and legend to Fig. 1 for details.

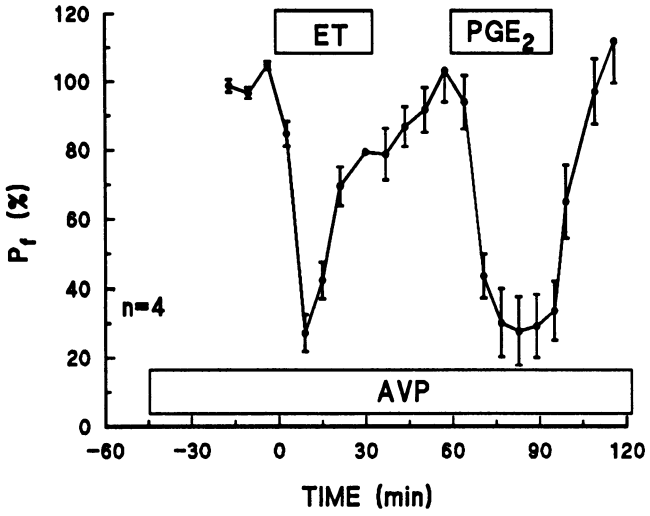

Figure 5. Absence of heterologous desensitization of ET effect. See text and legend to Fig. 1 for details.

and persistent inhibition of AVP-stimulated P_F in this segment (14). The above protocol was repeated, with the addition of PGE_2 (10^{-7}M) instead of ET during the second experimental period. Results are depicted in Fig. 5 and Table I. Despite desensitization of the ET effect, PGE_2-mediated inhibition of AVP-stimulated P_F was still manifest.

ET effects on $[Ca^{2+}]_i$

The signaling of agonists through PKC is often mediated via hydrolysis of phosphatidyl-inositol bisphosphate, which also results in the generation of IP_3 and subsequent IP_3-mediated increases in $[Ca^{2+}]_i$. To provide further evidence for coupling of ET via phosphatidyl-inositol bisphosphate hydrolysis in rat IMCD, we examined the effect of ET on $[Ca^{2+}]_i$. Results of representative experiments are depicted in Fig. 6. Addition of 10 nM ET to the bath of tubules preloaded with fura-2 resulted in a rapid spike-like increase in $[Ca^{2+}]_i$, from 20±7 to 360±89 nM ($n = 6$). The onset of this increase occurred within seconds of exposure to ET. After the initial spike $[Ca^{2+}]_i$ returned to stable baseline values within 2–4 min. In six tubules exposed to 1 nM ET, $[Ca^{2+}]_i$ rose from 36±8 to 102±22 nM, an increase significantly less than that seen with 10 nM ET ($P < 0.02$, unpaired t test). Thus the effect of ET on $[Ca^{2+}]_i$ is also dose dependent.

As was evident with ET effects on P_F, the ET effect on $[Ca^{2+}]_i$ also desensitized rapidly. Thus, as shown in Fig. 6, repeat exposure of tubules to ET was without effect on $[Ca^{2+}]_i$. That this desensitization is not due to a more generalized inability of IMCD cells to mobilize calcium is evidenced by the observation that PGE_2, added after tubule exposure to ET, still resulted in a biphasic calcium response, with both a transient peak and sustained increase in $[Ca^{2+}]_i$, identical to that previously described (14).

To determine whether the observed increase in $[Ca^{2+}]_i$ was due to mobilization of calcium from intracellular sites and/or influx of calcium across the basolateral membrane, the bath was changed to a nominally calcium-free solution containing 2 mM EGTA. As shown in Fig. 7, subsequent addition of ET to the bath resulted in the same pattern of increase in $[Ca^{2+}]_i$. Thus the ET-mediated increase in $[Ca^{2+}]_i$ likely represents release of calcium from intracellular sites, consistent with IP_3-mediated effects.

Since pertussis toxin pretreatment blocked ET-mediated inhibition of P_F and is also known to block ET and other receptor-coupled activation of phospholipase C (30–32), we determined whether ET-mediated changes in $[Ca^{2+}]_i$ in rat IMCD might also be sensitive to pertussis toxin. Perfused tubules were exposed to pertussis toxin using the same protocol as that for P_F experiments and then loaded with fura-2. As shown in Fig. 8, pertussis toxin pretreatment did not alter ET-mediated increases in $[Ca^{2+}]_i$. Since under the same conditions pertussis toxin pretreatment blocks ET-mediated inhibition of P_F (Table I), these data suggest that the ET-mediated increase in $[Ca^{2+}]_i$ during water flux studies is not sufficient to account for inhibition of P_F.

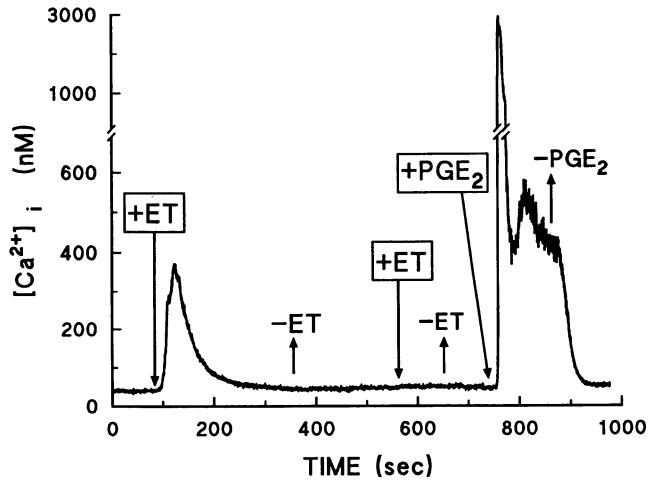

Figure 6. ET effect on ($[Ca^{2+}]_i$). Representative experiment demonstrating, from left to right, effect of 10 nM endothelin (*ET*) on $[Ca^{2+}]_i$ in microperfused rat IMCD, desensitization of the ET effect, and preservation of PGE_2-mediated increase in $[Ca^{2+}]_i$ despite ET desensitization. Time axis represents seconds from the beginning of data acquisition. Agents were added or removed as indicated by arrows.

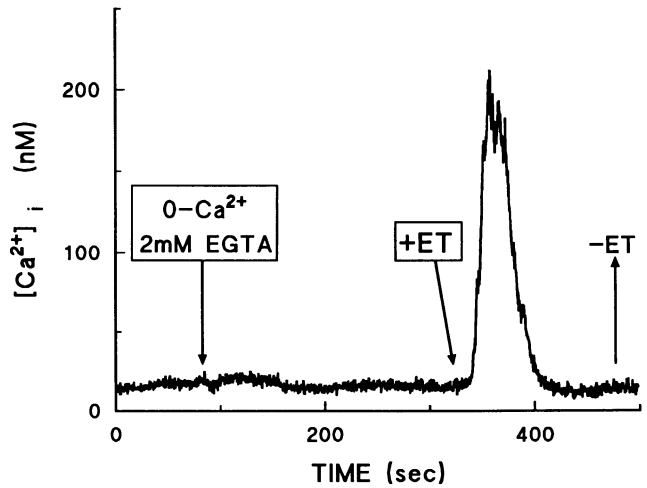

Figure 7. Effect of zero calcium bath on ET-stimulated calcium transient. Representative experiment demonstrating persistence of endothelin (*ET*, 10 nM) induced rise in $[Ca^{2+}]_i$ in a zero calcium, 2 mM EGTA bathing solution. See legend to Fig. 6 for details.

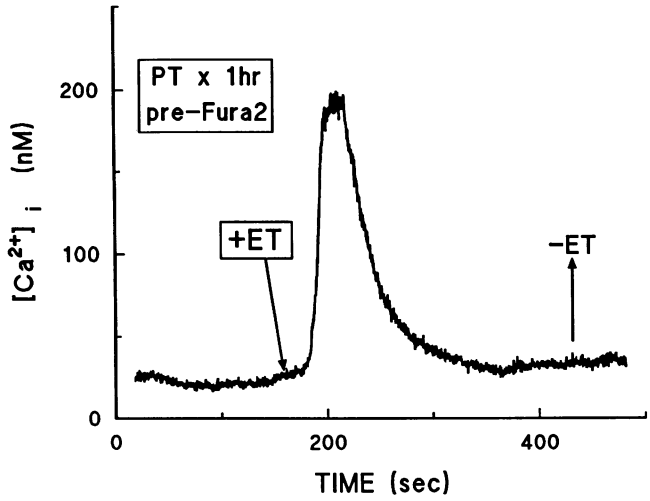

Figure 8. Persistence of ET (10 nM)-induced rise in $[Ca^{2+}]_i$ after pertussis toxin. Perfused tubules were treated with pertussis toxin (*PT*) for 1 h at 37°C before loading with fura-2. See legend to Fig. 6 for details.

Discussion

Using the in vitro tubule perfusion technique, these studies are the first to address in detail the signaling mechanisms for the direct inhibitory effect of ET on AVP-stimulated P_F in mammalian collecting duct. ET addition to the bathing solution of rat IMCDs that have already established a P_F response to AVP results in a rapid, marked inhibition of P_F. This effect is reversible and in fact desensitizes rapidly in continued presence of ET. ET has no effect on the water permeability response to the nonhydrolyzable cyclic AMP analogue 8-*p*-CPT-cAMP. The ET effect on AVP-stimulated P_F is completely blocked by the PKC inhibitor calphostin or by pretreatment with the G protein inactivator pertussis toxin, but not by the cyclooxygenase inhibitor indomethacin. ET causes an increase in $[Ca^{2+}]_i$ concentration in perfused IMCD, but the importance of this action for its inhibition of P_F is not certain.

A number of aspects of these studies warrant discussion: (*a*) comparison with other studies of modulation of AVP-dependent P_F in IMCD; (*b*) the site of ET effects; (*c*) the cell mechanisms involved in ET effects; (*d*) the desensitization to its effects; and (*e*) the physiological implications of these effects.

Comparison with other studies in rat IMCD

Our results corroborate and extend the observations of Oishi et al. (10), who were the first to describe the inhibitory effect of ET on AVP-stimulated P_F in rat IMCD. In the present studies, we found that the ET effect on P_F desensitized rapidly. Oishi et al. (10) averaged P_F values measured between 20 and 40 min after ET addition to the bath. Analysis of our data using P_F values obtained from up to four collections 22–45 min after addition of ET to the bath shows that ET results in a 27±6% decrease in AVP-stimulated PF ($n = 9$, $P < 0.01$). This inhibition is almost identical to the 24% inhibition observed by Oishi et al. (10) in experiments with 10 pM AVP and 10 nM ET. Thus, despite the desensitization we observed, inhibition of AVP-stimulated P_F by ET is similar in the two studies.

The dose–response relationship of P_F to AVP concentration in the present studies differs from that observed by others (17, 24, 33, 34). In this and previous studies we have consistently observed a lower AVP-independent P_F (13, 14). In the present study, we found that 5 pM AVP yielded close to a maximal P_F response, whereas the results of Star et al. (34), Nonoguchi et al. (24), and Lankford et al. (33) suggest that 10 pM AVP yields a submaximal response. The reason for these differences is uncertain. Lankford et al. (33) have shown that the diuretic state of rats influences AVP-independent P_F, but they did not examine its effect on AVP-dependent P_F. Our rats receive chow containing 196 meq/kg sodium, higher than the 56 meq/kg sodium in other studies (17, 24, 33, 34). We did not pretreat rats with furosemide whereas others have (17, 24, 33, 34). As summarized by Lankford et al. (33), large variability exists in AVP-dependent P_F in rat IMCD, even in studies from the same laboratory. Variability in dose responsiveness may vary similarly. Further studies are required to determine factors responsible for this variation.

Site of ET inhibition of AVP-stimulated P_F

Our observation that ET inhibits AVP-, but not cAMP-stimulated P_F suggests strongly that its functional effect occurs via alteration of cAMP metabolism. Our results using the cAMP analogue 8-*p*-CPT-cAMP corroborate the observations of Oishi et al. (10) who used dibutyryl cAMP. Since 8-*p*-CPT-cAMP is nonhydrolyzable, our experiments do not rule out the possibility of an ET effect on cyclic AMP phosphodiesterase activity. However, Tomita et al. (7) have shown that ET inhibits AVP-stimulated cyclic AMP accumulation in rat IMCD, even in the presence of phosphodiesterase inhibition. Taken together, our results suggest that ET inhibits P_F by inhibiting AVP-stimulated cyclic AMP generation.

Mechanism of ET inhibition of AVP-stimulated P_F

Role of endogenous PGs. In isolated rabbit medullary collecting duct cells, Zeidel et al. (6) demonstrated that ET inhibited Na/K-ATPase activity. This effect was blocked by cyclooxygenase inhibition and reproduced by exogenous PGE_2, suggesting an important role for ET-stimulated endogenous PG synthesis in mediation of ET effects in medullary tubules. In the present study, indomethacin did not block ET effects on AVP-stimulated P_F. Although our inability to define more precisely the time course of changes in P_F with ET may preclude detection of a small difference in the magnitude of ET inhibition of P_F in the absence or presence of indomethacin, our results show that inhibition of endogenous PG synthesis does not prevent functional effects of ET on P_F. Tomita et al. (7) also found that cyclooxygenase inhibition does not prevent ET-mediated inhibition of cAMP accumulation in rat IMCD. It is possible that PG synthesis by rat IMCD is modulated differently by ET than in rabbit IMCD or that signaling pathways are different between the two species (14). However, regardless of these possible differences, it is clear that the observed effect of ET on P_F is PG independent.

Role of PKC and cell calcium. As noted above, in most tissues examined, ET binding to its receptor is coupled to phosphatidyl-inositol bisphosphate hydrolysis, resulting in release of diacyl glycerol and activation of PKC, and generation of IP_3 with subsequent release of $[Ca^{2+}]_i$. Both the activation of PKC and the rise in $[Ca^{2+}]_i$ may mediate functional effects of ET (27). In the present studies, ET resulted in the intracellular release of Ca^{2+}, and its functional effect on P_F was blocked by the PKC inhibitor calphostin. Since calphostin acts at the regu-

latory domain of PKC, this antagonist is more specific than other described antagonists. We have previously used staurosporine to inhibit PKC (14), but given the ET effect on $[Ca^{2+}]_i$ and staurosporine's recognized effects on other kinases (35), especially calcium-calmodulin–dependent kinase, we chose to use the more specific antagonist. Tomita et al. (7) have demonstrated PKC dependence of ET inhibition of cyclic AMP accumulation. Taken together, our results are consistent with the hypothesis that ET-stimulated PKC activation is responsible for its inhibitory effects on P_F.

AVP-stimulated P_F was lower in tubules exposed to calphostin. However, in paired studies calphostin had minimal effects on an established permeability response to AVP. The lower stimulated P_F in the presence of calphostin may only reflect tubule-to-tubule variability (see above). Alternatively, the absence of a major calphostin effect when added after AVP could indicate a role for PKC in the development, but not maintenance, of the P_F response to AVP. Biochemical studies of PKC activity and cAMP generation would be necessary to distinguish these possibilities.

It is of interest to compare these results with those we obtained previously with PGE_2 in the same nephron segment (14). PGE_2 inhibits AVP-stimulated P_F in rat IMCD, at least partially at a post-cAMP site. This latter effect is sensitive to PKC inhibition. Why, then, does ET, which also couples via PKC, not inhibit P_F at a postcyclic AMP site? The answer may lie in another difference between PGE_2 and ET effects in IMCD: PGE_2 results in a steady state increase in $[Ca^{2+}]_i$ (14) whereas ET does not (see Fig. 6). It is possible that the steady state increase in $[Ca^{2+}]_i$, combined with PKC activation, is necessary for postcyclic AMP inhibition of P_F by PGE_2.

Studies in glomerular mesangial cells (27) and in mouse medullary collecting ducts (36) have demonstrated that ET causes a spike-like increase in $[Ca^{2+}]_i$ and a sustained elevation above baseline values. In the present studies, we did not observe this effect. Although species, tissue, or other technical details may be responsible for these differences, it is also possible that signaling mechanisms for ET are different in rat IMCD. Of note, our studies were performed in hypertonic media. In preliminary studies we have noted an inverse relationship between bath tonicity and $[Ca^{2+}]_i$, and similar observations have been made in cultured rat IMCD cells (37). Whether extracellular tonicity or sodium concentration or the basal, agonist-independent levels of $[Ca^{2+}]_i$ may alter subsequent response to ET remains to be determined. Similarly, it is possible that the low ambient $[Ca^{2+}]_i$ may modulate ET-stimulated PGE_2 production in these cells.

Role of pertussis toxin–sensitive G protein. Pretreatment of IMCD with pertussis toxin blocked ET-mediated inhibition of AVP-stimulated P_F. This provides strong evidence that ET signals via one of the guanine nucleotide regulatory proteins that act as substrate for pertussis toxin–catalyzed ADP-ribosylation. One class of these proteins, G_i, is thought to inhibit adenylate cyclase as a direct consequence of its receptor-mediated dissociation (31). Another class of G proteins, G_q, mediates activation of phospholipase C, and two types have been described, one sensitive to pertussis toxin and the other not (31). Which ones might be involved in our observed effects of ET? First of all, our results indicate that either pertussis toxin or calphostin alone blocks ET inhibition of P_F. This could indicate the existence of a single pathway involving both PKC and G_i. Preliminary evidence for such a process exists in a cultured mouse renal tubule cell line, where an α subunit of G_i regulates a PKC-dependent signaling pathway (38). Alternatively, ET may be coupled directly to both G_i and G_q, perhaps through different receptors, and both may be necessary for inhibition of P_F. In this regard, multiple receptors for ET have been described (2).

Secondly, since pertussis toxin does not alter ET-mediated increases in $[Ca^{2+}]_i$, the G_q involved in ET signaling is of the nonpertussis toxin–sensitive type. This pertussis toxin–insensitive coupling of ET to phospholipase C has been described in greater detail in cultured vascular smooth muscle cells (39). In rat mesangial cells in culture, a partial pertussis toxin sensitivity of ET-stimulated inositol phosphate release and $[Ca^{2+}]_i$ increase has been described (32). It is possible in our studies of individually perfused tubules, since each tubule is perfused under unique fura-2–loading conditions, light intensity, etc., that sensitivity to detect partial inhibition of $[Ca^{2+}]_i$ increases by pertussis toxin is lacking. Clearly, definition of signaling pathways for ET in IMCD will require further study.

An additional observation in the present study is that AVP-stimulated P_F was higher in tubules pretreated with pertussis toxin than in nonpertussis toxin–treated tubules. This effect is different than that observed in rabbit cortical collecting duct (40), where pertussis toxin blocks PGE_2-mediated inhibition of AVP-stimulated P_F but did not significantly alter AVP-stimulated P_F. The present findings suggest that endogenous factors exist in rat IMCD, and that these factors inhibit AVP-stimulated P_F in a pertussis toxin–sensitive manner. It is of interest in this regard that both ET and PGE_2 are synthesized by IMCD (9, 41). Whether these factors are activated by AVP or are present in unstimulated tubules is uncertain. It should be emphasized that interpretation of these results requires caution, given the inherent variability of tubule responsiveness.

Desensitization of ET effect

The reason for the rapid desensitization of ET effects remains unknown. It is possible that this phenomenon is only dose dependent and might not be seen at lower ET concentrations. Our experiments with PGE_2 show that cell processes involved in inhibition of AVP-stimulated P_F remain intact functionally even after exposure to ET. Since PGE_2-mediated inhibition of AVP-stimulated P_F is dependent on both phospholipase C and pertussis toxin–sensitive mechanisms (14, 25, 30), these results suggest that signaling pathways remain functional after exposure of tubules to ET. The data are consistent with the hypothesis that ET-specific processes are responsible for desensitization of its effects. Since definition of homologous desensitization should include comparison to effects of an agonist with similar signaling mechanisms, it should be noted that signaling mechanisms for PGE_2 are not identical to those for ET (see above). We have shown that a portion of the inhibitory effect of PGE_2 occurs at a postcyclic AMP site (14), and mechanisms involved in the precyclic AMP actions of PGE_2 in rat IMCD have not been entirely elucidated. However, other agonists and factors with well-described effects on AVP-stimulated P_F in rat IMCD inhibit P_F at a post-cAMP site (13, 24), so that no ideal agent for comparison exists presently.

With the above qualification, our results suggest that the desensitization to ET effects occurs via ET-specific processes. This homologous desensitization could occur by rapid down-

regulation of the ET receptor. Alternatively, ET might stimulate endogenous production of some substance or activation of some process that inhibits its effects, or that augments AVP-stimulated P_F. Since the desensitization persisted ≥30 min, this latter possibility would require continued production of this substance even in the absence of ET. In this regard, studies in cultured vascular smooth muscle cells have shown prolonged binding of ET to its receptor (39, 40) and prolonged ET-initiated activation of PKC after its removal from incubation solutions (41).

Another possibility is that inhibition of P_F and stimulation of $[Ca^{2+}]_i$ by ET could be mediated by an intermediary substance of which IMCD become exhausted early after exposure to ET. One possibility would be ET-stimulated PG production. However, indomethacin does not alter ET effects on P_F, suggesting that even the early effects of ET are not PG mediated. Finally, the $[Ca^{2+}]_i$ pool responsible for the ET-mediated increase in $[Ca^{2+}]_i$ may be limiting. If the rise in cell calcium is an important mediator of ET effects, this could also explain the transient nature of ET effects. This unexplained rapid desensitization to ET effects has also been described in cultured rat glomerular mesangial cells (42) and in cerebellar granular cells (43). The response of transporting epithelial cells such as those of the IMCD may be quite different than those of smooth muscle origin, and additional studies are necessary to elucidate the mechanism of this desensitization.

Physiological implications

AVP-stimulated P_F in IMCD is subject to regulation by numerous factors (13, 14, 22), some of which likely have important autocrine-like activities (13, 14). To date, the only locally produced substances shown to modulate renal concentrating capacity are PGs. However, evidence for synthesis of ET by IMCD cells now exists (9), and the present studies characterize a significant effect of this peptide on P_F. Thus, although factors that regulate endogenous production of ET by IMCD are not yet known, it is possible that ET represents another locally produced substance with important influence on renal water handling. It will be important to determine that the in vivo effect of ET does not desensitize as rapidly as in the present in vitro studies before an important physiological role of ET is defined. Preliminary studies by Schnermann et al. (5) suggest that the in vivo effect is indeed maintained.

In summary, these studies have documented that ET directly inhibits renal transepithelial transport, by characterizing its inhibitory effects on AVP-stimulated P_F in rat IMCD. These studies are also the first to address the signaling processes involved in this effect: inhibition of P_F occurs at a pre-cAMP site, is not dependent on endogenous PG synthesis, and involves signaling through PKC and pertussis toxin–sensitive mechanisms. Given that IMCD cells synthesize ET (9), ET may play an important autocrine function in the regulation of renal water excretion.

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