Luminal and basolateral endothelin inhibit chloride reabsorption in the mouse thick ascending limb via a Ca^{2+}-independent pathway

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1. The recent localization of endothelin synthesis and receptors in the thick ascending limb (TAL) prompted us to investigate a possible autocrine and/or paracrine effect of this agent. The net chloride flux (J_{Cl}) has been determined in isolated cortical and medullary TALs by the *in vitro* microperfusion technique.

2. In both segments, endothelin 1 (ET-1) at 10^{-8} M in the bath significantly decreased J_{Cl}, an effect which was partially reversible and observed at concentrations equal to or greater than 10^{-13} M.

3. This J_{Cl} inhibition (by 33·9 ± 3·2%) was blocked by BQ788 and was also observed with sarafotoxin 6_C and ET-3, indicating that endothelin receptor B (ET_B) are present in TAL.

4. ET-1 did not affect cAMP content under basal or hormone-stimulated conditions. The presence of a prostaglandin synthesis inhibitor also did not prevent the ET-1 action on J_{Cl}.

5. The ET-1-induced inhibition of J_{Cl} was prevented by protein kinase C inhibitors (staurosporine or GF109203) and was reproduced by diacylglycerol analogues (OAG and D_iC_8). However, ET-1 failed to increase intracellular Ca^{2+} concentration.

6. Addition of ET-1 or ET-3 to the apical surface induced a decrease of J_{Cl} through ET_B receptors, an effect which was not additive with that induced by basolateral ET-1, and was not concomitant with an increase in intracellular Ca^{2+} concentration.

7. It is concluded that the basolateral and luminal inhibitions of J_{Cl} by ET-1 in TAL, through ET_B receptors, is mediated by a protein kinase C activation which is independent of intracellular Ca^{2+} increase.

Since the discovery of endothelin as a potent endothelium-derived vasoactive peptide, three distinct isoforms have been identified to date, namely endothelin 1, 2, and 3 (ET-1, ET-2, and ET-3). Identification of the sites of production of these isoforms and evidence of their action outside the vascular system have furnished new insights on possible differences in their respective physiological roles (for review see Sokolovsky, 1995). In the kidney, recent studies (Ujiie, Terada, Nonogushi, Shinohara, Tomita & Marumo, 1992; Terada, Tomito, Nonogushi, Yang & Marumo, 1993) have localized ET-1 and ET-3 synthesis along the rat nephron: ET-1 is exclusively produced by glomeruli and inner medullary collecting tubules, whereas ET-3 is mainly synthetized in proximal tubules and outer medullary collecting tubules. A significant production of ET-3, but not of ET-1, has also been found in the medullary part (M) of the thick ascending limb (TAL) of the loop of Henle. It is of interest that early evidence suggested that ET-1 and ET-3 productions were controlled in different ways related to physiopathological states (Terada *et al.* 1993; Firth, Schricker, Ratcliffe & Kurtz, 1995).

Three subtypes of endothelin receptor have been cloned (ET_A, ET_B, and ET_C) (for review, see Sokolovsky, 1995). They all belong to the protein-G-coupled receptor family and differ from one another in their respective affinities for ligands: ET_A binds ET-1 and ET-2 with a 100-fold higher affinity than ET-3, ET_B displays similar affinities for the three peptides, whereas ET_C seems highly selective for ET-3. In the rat kidney, ET_A and ET_B are not equally distributed: mRNA coding for ET_A is present in glomeruli, vascular bundles, and interstitial cells, whereas mRNA coding for ET_B is found in glomeruli and tubular epithelium (Terada, Tomita, Nonogushi & Marumo, 1992; Chow, Subramanian,

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Nuovo, Miller & Nord, 1995). Furthermore, these studies pointed out that whereas ET_B mRNA is most abundant in inner medullary collecting tubules, small but detectable amounts have also been found in MTAL (Terada *et al.* 1992). No information about the cortical part (C) of TAL is available. In most target cells, ET_A and ET_B receptors are coupled to phospholipase C, triggering intracellular Ca^{2+} release and protein kinase C activation which are associated in some cases with the subsequent induction of phospholipases D and A_2. Inhibition of adenylyl cyclase has also been described (for review, see Sokolovsky, 1995).

In agreement with these biochemical studies, functional studies have shown that ET-1 plays a physiological role in renal function: it was reported to decrease the glomerular filtration rate in several ways including contraction of the glomerular mesangial cells and vasoconstriction of the afferent and efferent arteries (Badr, Murray, Breyer, Takahashi, Inagami & Harris, 1989). In contrast to these haemodynamic effects, there are also indications that ET-1 increases water and sodium excretion by a direct action on renal tubules. In the collecting duct, this effect has been ascribed, in part, to ET-1-induced inhibition of the antidiuretic hormone (ADH)-stimulated cAMP generation and, consequently, of the ADH-induced increase in water permeability (Tomita, Nonogushi & Marumo, 1991; Nadler, Zimpelmann & Hébert, 1992; Tomita, Nonogushi, Terada & Marumo, 1993).

Since NaCl reabsorption in TAL contributes to the urinary dilution–concentration process, the question arises as to whether the natriuretic and diuretic effects of endothelin could be accounted for, in part, by an inhibitory action on the TAL reabsorptive capacity. The purpose of this work was, therefore, to evaluate the possible effect of endothelin on net Cl^- flux (J_{Cl}) in both MTAL and CTAL. Moreover, since endothelin synthesis probably occurs in TAL, we investigated whether this agent is active at both the luminal and basolateral surfaces of the tubular cells.

METHODS

Microperfusion experiments

CTALs and MTALs were microperfused *in vitro* following the technique first described by Burg (Burg, Grantham, Abramow & Orloff, 1996) and routinely used in our laboratory. Animals were bred and killed according to the guidelines from the Ministère de l'Agriculture et de la Pêche (authorization no. 5443). Briefly, male Swiss mice weighing 18–20 g were killed by cervical dislocation and exsanguinated. Coronal slices were cut from both kidneys and immediately immersed in a cold perfusion solution (for composition see below) with 0·1% bovine serum albumin added. CTAL and MTAL were dissected from the medullary rays of the cortex and from the inner strip of the outer medulla, respectively. Each tubule was then transferred to a Lucite chamber thermostatically maintained at 36·0 ± 0·1 °C, with a flow rate of ~5 ml min^{-1}.

Each perfused tubule was allowed to equilibrate for 1 h for CTAL and 0·5 h for MTAL. After a 30 min control period the agent under study was added to the bath or to the lumen, according to the experimental protocol. After 10 min, a 30 min experimental period followed. For recovery experiments, an additional 30 min experimental period was performed after removal of the agent. The luminal fluid was collected every 10 min. When agents were present on the luminal side, the luminal fluid was manually changed with a syringe, under microscopic observation, as we have already reported (Néant & Bailly, 1993). This technique was found not to modify the J_{Cl} in control tubules.

The composition of the perfusion solution was as follows (mM): NaCl, 140; K_2HPO_4, 2·4; NaH_2PO_4, 0·6; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; urea, 10. Glucose (5 mM) was added to the bathing solution. All solutions were adjusted to pH 7·39–7·42.

Chloride concentrations in collected fluid (C_c) and perfusate (C_p) were determined by microelectrometric titration. The tubular flow rate (V) was calculated from the volume of the collected sample, assuming that water reabsorption was negligible. The length (L) of the perfused tubule was measured with an eyepiece micrometer at × 400 magnification. The net chloride flux was calculated as $J_{Cl} = (C_c - C_p)V/L$, expressed in pmol min^{-1} (mm tubular length)$^{-1}$.

Data from the three collections of each 30 min period were pooled and considered as one datum. Values are expressed as means ± S.E.M. Statistical significance was evaluated within each series by Student's paired t test. For comparing the different series, results were expressed as the percentage of J_{Cl} inhibition for each tubule since a linear relationship exists between J_{Cl} in the control and the experimental period (see Results). Statistical treatment was the one-way analysis of variance followed by Fisher's least significant difference test, using as the parameter the difference of $\log(J_{Cl})$ between the control and experimental periods.

Determination of intracellular calcium concentration ($[Ca^{2+}]_i$)

Experiments were carried out on isolated tubules, according to the technique used in our laboratory (Champigneulle, Siga, Vassent & Imbert-Teboul, 1993). Briefly, tubules were dissected from eight male Swiss mice (18–20 g body weight), following the protocol described above for microperfusion experiments. Each tubule was then transferred onto a slide where it was loaded for 1 h at room temperature in the dark, with a solution containing fura-2 AM (acetomethoxy ester; 10^{-5} M) in DMSO (final dilution 500). Each fura-2-loaded tubule was then transferred to a Lucite chamber, held tightly by pipettes at both sides, and superfused at 36 °C at a rate of 10–12 ml min^{-1}, so that total equilibrium in the chamber was achieved in about 15–20 s. The bathing solution contained (mM): NaCl, 140; KCl, 5; $NaHCO_3$, 4; NaH_2PO_4, 0·44; Na_2HPO_4, 0·33; $MgCl_2$, 1; $MgSO_4$, 0·8; $CaCl_2$, 1; glucose, 2; Hepes, 10; pH 7·4. When ET-1 was added on the apical side, the tubules were microperfused as described for J_{Cl} determinations (see above) and the luminal fluid was manually changed with a syringe. This manoeuvre could be made rapidly since with no sampling required there was no oil in the collecting pipette and rapid luminal fluid change did not provoke tubule swelling. For each tubule, measurements of fura-2 fluorescence were carried out after a 5–10 min equilibration period.

The portion of the tubule selected for fluorescence measurements was centered across a circular field diaphragm (60 mm diameter) and included about twenty to thirty cells. Fluorescence intensities were recorded at two excitation wavelengths, 340 and 380 nm. Tubule autofluorescence at the two wavelengths was determined on homologous segments not loaded with fura-2 and superfused as described above. For each tubule, the net fluorescence intensities of fura-2 at 340 and 380 nm were obtained by subtracting the corresponding autofluorescence from all measurements. $[Ca^{2+}]_i$ was

calculated from the ratio R of the fluorescence intensities at 340 and 380 nm according to the following equation:

$$[Ca^{2+}]_i = K_d (R - R_{min}/R_{max} - R)(S_{f2}/S_{b2}),$$

where K_d (224 nM) is the dissociation constant of fura-2 for calcium; R_{min} and R_{max} are the values of R at 0 and the saturating calcium concentration, respectively; (S_{f2}/S_{b2}) are the fluorescence intensities at 380 nm at 0 and the saturating calcium concentration, respectively.

cAMP content

cAMP content was determined by radioimmunoassay in tubules microdissected after collagenase dissociation of kidney tissue, following a procedure currently used in our laboratory (Firstov, Aarab, Mandon, Siaume-Perez, De Rouffignac & Chabardes, 1995). Twenty-one male Swiss mice were anaesthetized with sodium pentobarbitone (0·1 mg (10 g body wt)$^{-1}$). The left kidney was perfused *in situ* via the abdominal aorta with 5 ml of incubation solution containing (mM): NaCl, 120; KCl, 5; $CaCl_2$, 1; $MgSO_4$, 1; Na_2HPO_4, 4; $NaHCO_3$, 4; glucose, 5; CH_3COONa, 10; and Hepes, 20. Dextran 40 000 (0·3% w/v), collagenase (151 U mg^{-1}) 0·3% w/v, and BSA 0·1% w/v were added. Thin pyramids were excised along the cortico-papillary axis of the left kidney and were then incubated for 10–15 min at 35 °C in the incubation solution containing 0·1% collagenase.

For cAMP determinations, one or two tubule segments were transferred to a slide in 2 ml of incubation solution with 5×10^{-6} M Ro 201724, an inhibitor of the high affinity cAMP phosphodiesterase (type IV). Individual segments were photographed for subsequent determination of their lengths. Each sample was preincubated for 10 min and then incubated for 4 min at 35 °C in the presence of 2 μl of either the solution alone (basis) or the relevant hormone. The reaction was stopped by transferring the sample into a tube containing 25 μl of a mixture of formic acid and absolute ethanol (5% v/v). After evaporation, acetate buffer was added and cAMP was determined by radioimmunoassay after acetylation. The limit of detection was one femtomole of cAMP per tube.

Five to eight replicates were determined for each experimental condition. The mean of these replicate values was considered as one datum and was used to calculate the mean and standard error of the response in several animals. Statistical significance was evaluated by the one-way analysis of variance followed by Fisher's least significant difference test.

Materials

ET-1 and ET-3 were purchased from Alexis Co. (Laüfalfingen, Switzerland), GF 109203 and BQ 788 from RBI (Natick, MA, USA), OAG (1-oleoyl, 2-acetyl-*sn*-glycerol) and D_iC_8 (1,2-dioctanoyl glycerol) from Calbiochem (La Jolla, CA, USA). cAMP antibodies were obtained from INSERM 64 (Hôpital Tenon, Paris, France). All other compounds were purchased from Sigma.

RESULTS

Effects of basolateral and luminal endothelin on Cl^- reabsorption

Addition of 10^{-8} M ET-1 to the bath, significantly decreased J_{Cl} in MTAL (Fig. 1). This inhibitory effect (33·1 ± 4·2%) was significantly higher than that observed in time control tubules (4·7 ± 2·5%; $P < 0·001$). A similar effect was observed in CTAL, in which ET-1 significantly decreased J_{Cl} by 35·9 ± 4·0% (Fig. 1). This effect of ET-1 on J_{Cl} resulted from a significant reduction of the chloride concentration gradient between the perfused and collected fluids (10·7 ± 1·4 *vs.* 14·7 ± 1·6 mM, $P < 0·001$, and 15·7 ± 5·3 *vs.* 24·4 ± 7·8 mM, $P < 0·05$, in MTAL and CTAL, respectively) while the tubule flow rates did not change significantly (3·2 ± 0·3 *vs.* 3·4 ± 0·3 nl min^{-1}, and 3·2 ± 0·6 *vs.* 3·2 ± 0·6 nl min^{-1}, n.s., for MTAL and CTAL, respectively). The variability of J_{Cl} between individual tubules did not invalidate the overall effect since: (1) each tubule was used as its own control, and (2) a significant linear relationship existed between the J_{Cl} in the

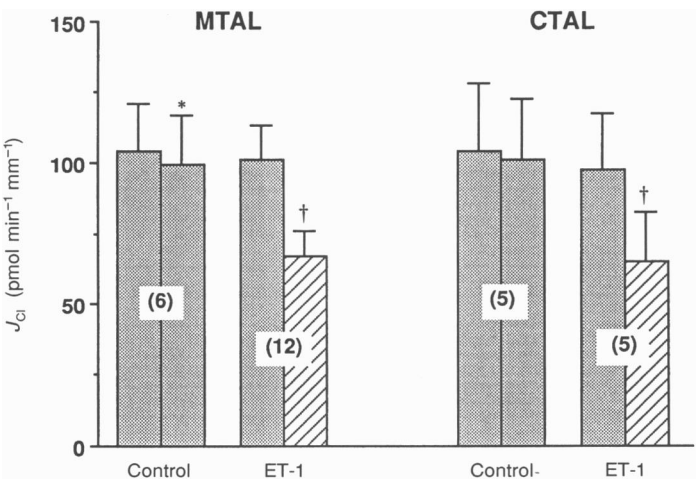

Figure 1. Inhibitory effects of endothelin on net chloride reabsorption in MTAL and CTAL

After a control period (stippled bars), the bath either remained unchanged, for time control tubules (Control), or 10^{-8} M endothelin 1 (ET-1, hatched bars) was added. (n), number of tubular samples. Significantly different from the preceding period: * $P < 0·05$; † $P < 0·001$.

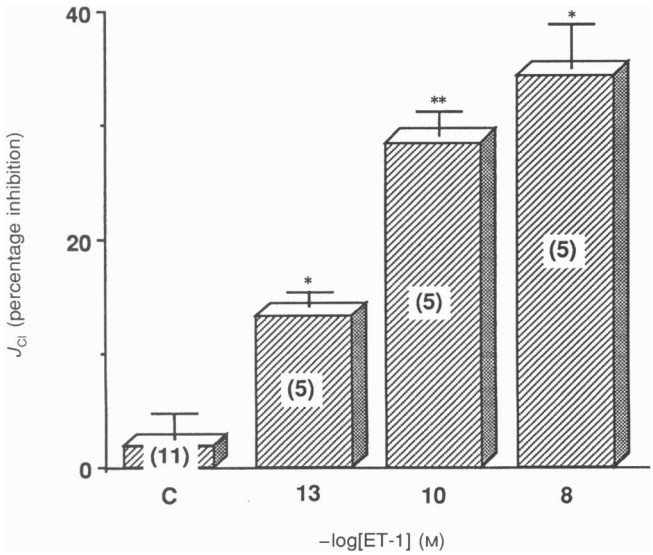

Figure 2. Inhibitory effects of different concentrations of endothelin 1 on net chloride reabsorption

The ordinate represents the decrease of J_{Cl} in the experimental period as a percentage of the control value, determined in 4 separate groups of TALs. C, control group, (n), number of tubules. Significance of difference from the preceding value: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

experimental period (y) and that in the control period (x). This is shown in either the presence of endothelin ($y = 0{\cdot}70x - 0{\cdot}68$, $r = 0{\cdot}87$, $P < 0{\cdot}001$; pooled data from ET-1 and ET-3, see below) or in time-control tubules ($y = 0{\cdot}94x + 2{\cdot}51$, $r = 0{\cdot}98$, $P < 0{\cdot}001$). Moreover, the slopes of these regression lines were significantly different ($P < 0{\cdot}02$), indicating that the relative effect of ET-1 was independent of the basal J_{Cl} value over the range measured in these experiments. Since the responses of MTAL and CTAL to ET-1 were similar with regard to J_{Cl}, cAMP and $[Ca^{2+}]_i$ (see below), the data have been obtained indiscriminately from one or the other segment, and classified as TAL, in the following experiments.

When concentrations of ET-1 from 10^{-13} M to 10^{-8} M were tested on separate groups of tubules, a dose-dependent effect was observed (Fig. 2). Endothelin 1-induced inhibition of J_{Cl} was reversible as J_{Cl} increased significantly by $21{\cdot}4 \pm 3{\cdot}4\%$ after removal of the agent (from $76{\cdot}5 \pm 10{\cdot}6$ to $91{\cdot}0 \pm 11{\cdot}1$ pmol min^{-1} mm^{-1}, $n = 8$, $P < 0{\cdot}001$, Fig. 3). However, when the control and recovery periods were performed during individual experiments (Fig. 3), the reversibility was found to be only partial, since the J_{Cl} remained significantly ($P < 0{\cdot}01$) lower after ET-1 removal compared with initial values ($105{\cdot}0 \pm 10{\cdot}6$, $68{\cdot}9 \pm 11{\cdot}9$, and $82{\cdot}1 \pm 12{\cdot}0$ pmol min^{-1} mm^{-1}, before, during, and after the presence of ET-1, respectively).

The diminution of J_{Cl} induced by 10^{-8} M ET-1 (Fig. 4, column 2) was prevented by BQ788, a selective antagonist of the ET_B receptor (Fig. 4, column 3, from $103{\cdot}7 \pm 19{\cdot}5$ to $97{\cdot}4 \pm 16{\cdot}8$ pmol min^{-1} mm^{-1}; n.s.). This inhibitory effect of ET-1 (10^{-8} M) was also obtained by the presence of 10^{-11} M ET-3 (Fig. 4, column 4, from $133{\cdot}6 \pm 24{\cdot}0$ to $98{\cdot}9 \pm 18{\cdot}0$ pmol min^{-1} mm^{-1}, $P < 0{\cdot}02$), and 10^{-8} M sarafotoxin 6_C, a specific agonist of the ET_B receptor, (Fig. 4, column 5, from $85{\cdot}5 \pm 18{\cdot}8$ to $61{\cdot}1 \pm 17{\cdot}7$ pmol min^{-1} mm^{-1}).

Apical addition of ET-1 (10^{-8} M) induced a $35{\cdot}1 \pm 7{\cdot}6\%$ inhibition of J_{Cl} (Fig. 5, column 1). This effect was also seen with the same concentration of ET-3 ($33{\cdot}2 \pm 4{\cdot}2\%$

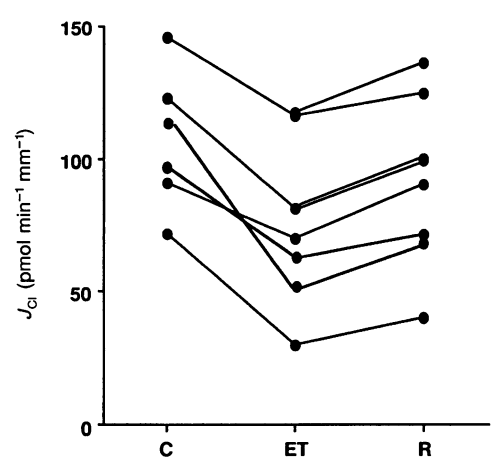

Figure 3. Reversibility of the endothelin effect on net chloride reabsorption, in individual TALs

After a control period (C), endothelin 1 (ET) was added to the bath at a concentration of 10^{-8} M, and then removed from the bath (R).

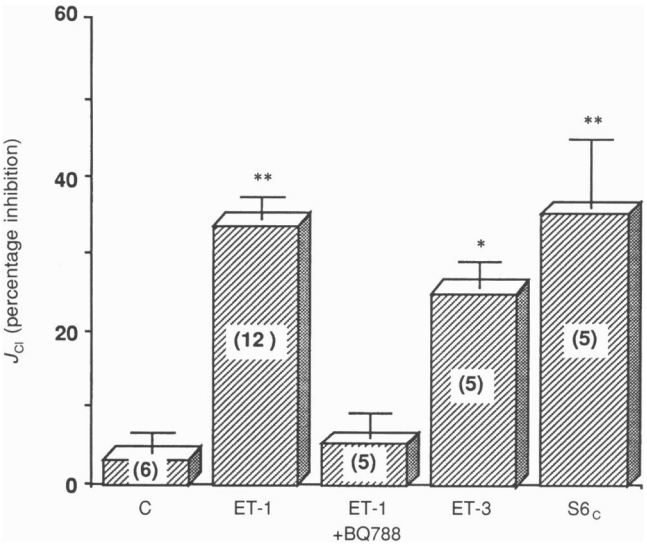

Figure 4. Effects of endothelin agonists and antagonists on net chloride reabsorption, in TAL

The ordinate represents the decrease of J_{Cl} in the experimental period as a percentage of the control value. All agents were added to the bath, either during the experimental period in the case of endothelin 1 (ET-1, 10^{-8} M), endothelin 3 (ET-3, 10^{-11} M), and sarafotoxin 6_C ($S6_C$, 10^{-8} M), or from the control period onwards in the case of BQ788 (10^{-7} M). (n), number of tubules. Significantly different from the control plus ET-1 + BQ788 values: * $P < 0{\cdot}05$, ** $P < 0{\cdot}001$.

inhibition, Fig. 5, column 2). The inhibitory effect of endothelin was eliminated by a simultaneous luminal administration of BQ788 ($2{\cdot}6 \pm 3{\cdot}3\%$ inhibition, Fig. 5, column 3) whilst addition of the receptor antagonist to the bath was without effect ($34{\cdot}5 \pm 6{\cdot}1\%$ inhibition, Fig. 5, column 4). Simultaneous additions of endothelin to the bath and the perfusate (Fig. 5, column 5) failed to induce a greater effect ($28{\cdot}9 \pm 3{\cdot}2\%$) than that elicited by the agent present on only one or the other of the two sides.

In the presence of 3×10^{-6} M Ibuprofen, an inhibitor of constitutive and inducible cyclo-oxygenase activities, ET-1 significantly decreased J_{Cl} by $29{\cdot}3 \pm 4{\cdot}0\%$ ($n = 5$), a value similar to that obtained in the absence of prostaglandin synthesis inhibitor.

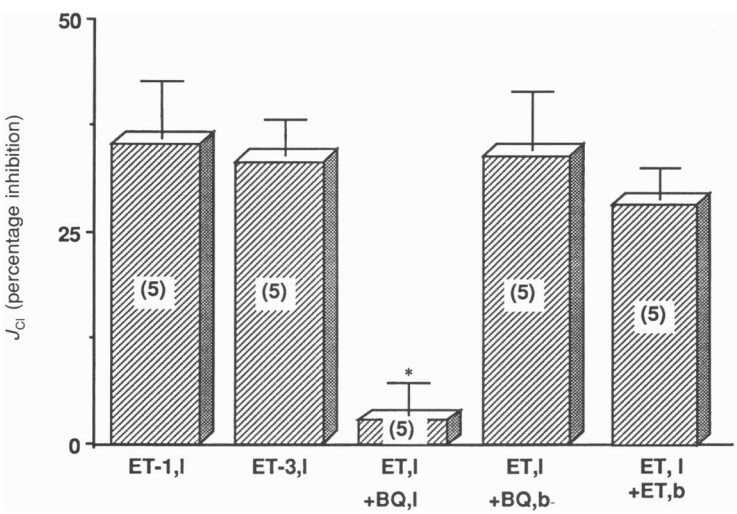

Figure 5. Effect of luminal endothelin 1 or 3 on net chloride reabsorption, in TAL

Endothelin was used at a concentration of 10^{-8} M for both ET-1 and ET-3 in the lumen (ET-1,l and ET-3,l). BQ788 (10^{-7} M) was added either to the lumen (BQ,l) simultaneously with ET (ET-1 or ET-3) during the experimental period, or to the bath (BQ,b), from the control period onwards. ET,l + ET,b: endothelin was added simultaneously to the bath and perfusate. * Significantly different ($P < 0{\cdot}05$) from the other groups.

Table 1. cAMP contents of isolated thick ascending limbs and cortical collecting ducts

	TAL	*n*	CCD	*n*
Basis	3·7 ± 0·5	(15)	5·8 ± 2·0	(5)
ET-1	7·6 ± 2·4	(8)	5·9 ± 3·3	(4)
ADH	23·7 ± 3·8*	(13)	66·3 ± 9·6	(6)
ADH + ET-1	26·1 ± 5·8*	(10)	21·4 ± 6·4†	(6)
ADH + PGE_2	6·6 ± 0·7	(5)	n.t.	

cAMP content measured in fmol $(4\ min)^{-1}\ mm^{-1}$. Values are means ± S.E.M. (*n*), number of experiments. For each experiment, 5 to 8 tubule samples per condition were tested. Cyclic AMP accumulation was determined in the presence of Ro 201724, an inhibitor of the phosphodiesterases IV. ET-1, endothelin 1, 10^{-7} M; ADH, 8 arginine vasopressin, 10^{-8} M; PGE_2, prostaglandin E_2, 3×10^{-7} M. n.t., not tested. * $P < 0{\cdot}001$ relative to the basal value, † $P < 0{\cdot}01$ relative to ADH.

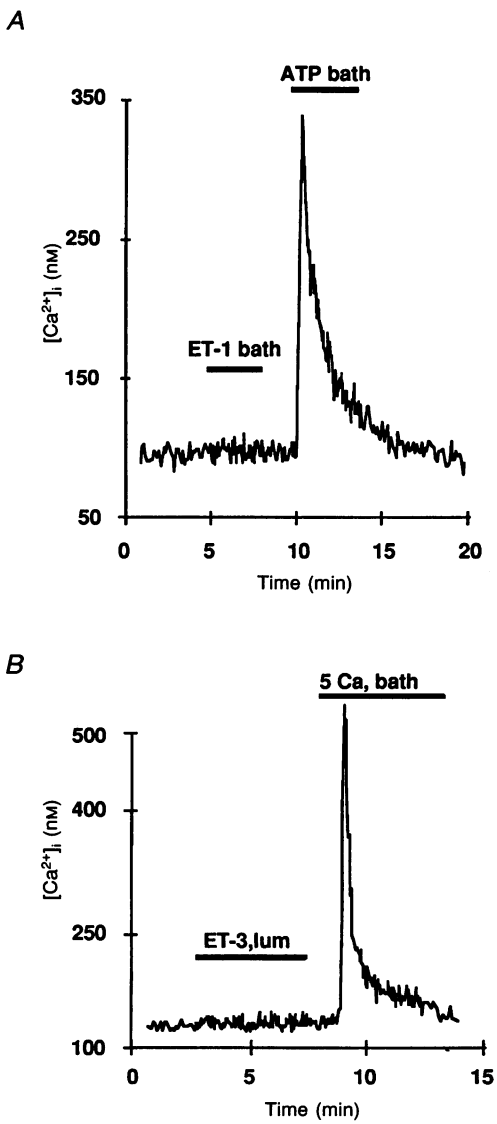

Figure 6. Recordings from two representative experiments of determination of intracellular calcium concentration ($[Ca^{2+}]_i$), in TAL

A, endothelin 1 (ET-1, 10^{-8} M) and adenosine triphosphate (ATP, 10^{-4} M) were present in the bath. *B*, 5 mM calcium (5 Ca) was present in the bath; endothelin 3 (ET-3, 10^{-8} M) was present in the lumen (lum).

Table 2. Role of protein kinase C in the endothelin effect on net chloride reabsorption, in TAL

	J_{Cl} (pmol min^{-1} mm^{-1})		Percentage inhibition
	Control	Experimental	
Bath			
ET + GF (5)	133·9 ± 18·2	124·0 ± 16·9*	7·4 ± 2·2
ET + STP (5)	179·3 ± 41·0	166·1 ± 37·1**	6·8 ± 1·2
OAG/ D_1C_8 (11)	156·3 ± 18·2	109·7 ± 15·6***	31·7 ± 4·4†
Lumen			
ET + GF (5)	111·1 ± 15·4	103·5 ± 13·5*	6·1 ± 1·6

Values are means ± S.E.M. (n), number of tubule samples. J_{Cl}, net chloride reabsorption. ET, endothelin 1 or 3, added at a concentration of 10^{-8} M to either the bath (rows 1 and 2) or the lumen (row 4), during the experimental period. Protein kinase C inhibitors, GF109203 or staurosporine (STP), were present at a concentration of 10^{-7} M in either the bath (rows 1 and 2) or the lumen (row 4) from the control period onwards. OAG and D_1C_8 were added to the bath at a concentration of 10^{-8} M during the experimental period. Within each group (one row), differences in J_{Cl} between control and experimental periods were compared by Student's paired t test (* $P < 0·05$, ** $P < 0·01$ and *** $P < 0·001$); between the groups (columns), the percentages of inhibition were compared by ANOVA († $P < 0·001$ when compared with the three other groups).

Absence of an endothelin effect on the cAMP pathway

Accumulation of cAMP was unaffected by ET-1 under either basal or ADH-stimulated conditions (Table 1). This lack of an ET-1 effect was shown not to be due to damaged tubules or to altered hormone solutions. As expected, the ADH-stimulated cAMP accumulation was inhibited by PGE_2 in the TAL, and by ET-1 in the cortical collecting duct. In addition, the effect of ET-1 on J_{Cl} was not potentiated by the presence of glucagon, which like ADH stimulates adenylyl cyclase activity in TAL (Morel, Chabardès, Imbert-Teboul, Le Bouffant, Hus-Citharel & Montégut, 1982) since there was a 28·5 ± 5·3% inhibition ($n = 7$) in the presence of glucagon and 33·9 ± 3·2% ($n = 17$, n.s.) in its absence.

Transduction pathway involved in the effect of endothelin on J_{Cl}

There was no effect of the addition of basolateral ET-1 when protein kinase C inhibitors (staurosporine or GF 109203) were also present at a concentration of 10^{-7} M, from the control period onwards (Table 2). Indeed, the slight decrease of J_{Cl} observed in the presence of protein kinase C inhibitors was not significantly different from that observed in time-control experiments (3·0 ± 2·4%). However, the inhibitory effect of basolateral ET-1 on J_{Cl} was also seen with 10^{-6} M of the diacylglycerol analogues OAG or D_1C_8, since addition to the bath of either of these agents reduced J_{Cl} by 31·7 ± 4·4%.

Inhibition of J_{Cl} by luminal ET-3 was abolished by previous luminal addition of GF109203 (6·1 ± 1·6% inhibition, Table 2). That this experimental procedure did not impair tubular function was verified: in two tubules, the presence of the protein kinase C inhibitor failed to stop a further inhibitory effect of luminal cyclic GMP (35·9 and 23·3% inhibition; Néant & Bailly, 1993). It is noteworthy that the presence of GF109203 in the bath failed to suppress the luminal effect of ET-3 (41·0 ± 7·5% inhibition, $n = 4$), although it reduced the basolateral effect of the hormone in the same tubule.

To determine whether ET-1 stimulates protein kinase C through activation of phospholipase C, the effect of ET-1 on cytosolic calcium was evaluated. In TAL, $[Ca^{2+}]_i$ remained unchanged after addition of 10^{-8} M ET-1 to the bath ($n = 16$, Fig. 6*A*). As a test of tubule viability, $[Ca^{2+}]_i$ responses were measured following activation of calcium sensors by peritubular addition of 5 mM calcium (Paulais, Baudoin-Legros & Teulon, 1996) or of purinoreceptors by 10^{-4} M ATP (Paulais, Baudoin-Legros & Teulon, 1995) both of which increased $[Ca^{2+}]_i$ by 273 ± 41 nM ($n = 11$) and 254 ± 66 nM ($n = 5$), respectively. It should be noted that in the rat also, endothelin failed to increase $[Ca^{2+}]_i$ although a peak could be provoked by addition of 10^{-8} M angiotensin II (data not shown). Moreover, as a further control, it was verified that ET-1 elicited a $[Ca^{2+}]_i$ increment of 172 ± 54 nM ($n = 5$) in the mouse cortical collecting duct.

Similarly, 10^{-8} M ET-3 did not alter $[Ca^{2+}]_i$ when added to the luminal side of TAL ($n = 5$, Fig. 6*B*). With the same tubule, however, increasing the bath calcium concentration from 1 to 5 mM evoked a $[Ca^{2+}]_i$ peak of 204 ± 32 nM above the basal value.

DISCUSSION

The results of this study demonstrate that endothelin, whether added luminally or basolaterally, inhibits the reabsorptive function of MTAL and CTAL. In contrast to mechanisms characterized in other target cells, the

transduction pathway in these kidney cells does not involve inhibition of cAMP production but is related to activation of a protein kinase C which, however, is independent of increases in intracellular calcium.

Effect of endothelin on J_{Cl}

The decrease of J_{Cl} observed in the presence of ET-1 is unlikely to result from a toxic action of the peptide as this effect is inhibited by the use of a specific antagonist. Relevant to the partial reversibility of the effect of endothelin are the very slow rates of dissociation of receptor–ligand complexes reported in many structures (for review see Sokolovsky, 1995), including renal papillary membranes (Martin, Marsden, Brenner & Ballermann, 1989). Our study presents pharmacological evidence to indicate that the ET-1-induced inhibition of J_{Cl} occurs through the ET_B receptor. Moreover, the fact that J_{Cl} inhibition can be induced by ET-1 precludes the involvement of ET_C receptors. Terada *et al.* (1992) observed a small signal for the ET_B receptor whereas other workers did not detect ET_A or ET_B receptor mRNA (Chow *et al.* 1995). Our study confirms the presence of functional ET_B receptors in the mouse CTAL and MTAL and also emphasizes either species differences or a translation efficiency sufficient to allow protein expression from very low levels of mRNAs in this segment. It should be noted that mRNAs encoding the ET_B receptor have not been detected in the proximal tubule (Terada *et al.* 1992), yet ET-1 action in this segment is well-documented (Garcia & Garvin, 1994; Guntupalli & DuBose, 1994; Walter, Helmle-Kolb, Forgo, Binswanger & Murer, 1995) and is shown to be partly linked to activation of the ET_B receptor (Knotek, Jaksic, Selmani, Skoric & Banfic, 1996). It must be kept in mind, however, that although mRNA encoding ET_C has not been detected in murine species this is because this receptor has not yet been cloned in mammals.

A significant inhibitory effect of ET-1 in TAL was observed at a concentration of 10^{-13} M, which suggests that this action is of physiological relevance since: (i) the threshold for proximal tubule effects is 10^{-13} M (Garcia & Garvin, 1994) which is three orders of magnitude lower than the threshold found in the collecting duct (Tomita *et al.* 1991), both segments being well-recognized target sites of endothelin; and (ii) detectable amounts of ET-3 and ET-1 have been found in rat renal medulla, under basal conditions for the former (Terada *et al.* 1993) and following stimulation by inflammatory agents for the latter (Ujiie *et al.* 1992). Thus these results, taken together, strongly suggest that ET-3 and ET-1 act as autocrine and/or paracrine agents in TAL.

Transduction pathway involved in the endothelin effect in TAL

The present data indicate that the ET-1 inhibitory effect on J_{Cl} in TAL is not coupled to an inhibition of cAMP production. It may be assumed that both basolateral and apical ET-1 fail to impair generation of this nucleotide, because the luminal surface is presumably accessible to ET-1 in cAMP experiments as firstly, microscopic observation revealed that most microdissected tubules displayed a fairly open lumen, and secondly, numerous studies report that electrolyte transport through the apical Na^+–K^+–$2Cl^-$ cotransporter in MTAL suspensions is inhibited by incubation with selective blockers such as furosemide and bumetanide, suggesting that they can freely reach the luminal side of the tubules. The lack of effect of ET-1 on cAMP production in mouse TAL is in agreement with a previous report by Tomita (Tomita, Nonogushi & Marumo, 1990) concerning the rat nephron. In this latter study, however, cAMP content was determined while phosphodiesterase activity was fully inhibited by IBMX, indicating that endothelin fails to modify adenylate cyclase activity. The present experiments, in which phosphodiesterase activity was partially preserved, show that endothelin is unlikely to act on cAMP degradation. The observation that in TAL ET-1 did not affect cAMP production shows that the process here differs from that of the collecting duct in which ET-1 prevented the ADH-stimulated increase in water permeability at the cAMP generation step (Tomita *et al.* 1991; Nadler *et al.* 1992).

In many target cells, including renal medullary interstitial cells (Wilkes *et al.* 1991) and inner medullary collecting duct cells (Kohan, Padilla & Hughes, 1993), endothelin has been shown to generate arachidonic acid and metabolites including PGE_2 which may explain ET-1 actions on TAL. However, this is unlikely because (i) the ET-1 effects were unchanged after cyclo-oxygenase inhibition, and (ii) both arachidonic acid and PGE_2 are known to attenuate ADH-stimulated cAMP accumulation in this segment (Torikai & Kurokawa, 1983; Firstov *et al.* 1995).

The results presented here provide evidence for the activation of protein kinase C by endothelin. Surprisingly, administration of the phorbol ester PMA at a concentration of 10^{-7} M does not alter J_{Cl} in mouse CTAL, as already reported by us (Néant, Imbert-Teboul & Bailly, 1994). A similar anomaly has been observed for proximal tubule, in that PMA failed to produce the ET-1-induced increase in the Na^+–H^+ exchanger although this effect was consistently suppressed by protein kinase C inhibitors (Guntupalli & DuBose, 1994; Walter *et al.* 1995). The presence of other effects of phorbol esters in addition to protein kinase C activation may explain these discrepancies.

There is clear evidence from this work that basolateral or luminal ET-1 does not stimulate $[Ca^{2+}]_i$ increases in TAL, in contrast to its observed effect made in collecting tubules (Naruse, Uchida, Ogata & Kurokawa, 1991, and our results). This lack of an ET-1 effect on $[Ca^{2+}]_i$ is in agreement with the report concerning basolateral ET-1 in mouse TAL (Naruse *et al.* 1991). To our knowledge, the present results show for the first time a functional effect of endothelin at a target site where the hormone does not evoke intracellular calcium mobilization. This absence of endothelin-induced $[Ca^{2+}]_i$ increase probably excludes a possible activation of the so-called classical protein kinase C isoforms, as a

consequence of phospholipase C stimulation. Rather, the results afford evidence that endothelin action is mediated by the so-called novel protein kinase C isoforms, the activation of which requires diacyl glycerol but not $[Ca^{2+}]_i$ increases. Moreover, the recent demonstration in the renal outer medulla of several protein kinase C isoforms, including an atypical one independent of calcium and phorbol ester (Östlund *et al.* 1995), raises the possibility that endothelin activates such a protein kinase C, in addition to the phorbol ester-activated isoforms. The mechanism underlying protein kinase C activation requires further investigation of the direct stimulation of phospholipase D by endothelin. Some data show that ET-1 induces phospholipase D stimulation in renal structures (Wilkes *et al.* 1991; Baldi, Musial & Kester, 1994). In these studies, however, phospholipase D was activated by protein kinase C and was, thereby, primarily linked to phospholipase C induction.

Polarization of the endothelin effect on J_{Cl}

It is interesting that endothelin inhibits J_{Cl} when it is added on either the basolateral or the luminal side of the tubule. The fact that the TAL epithelium is tight and that the apical effect of endothelin occurs in the presence of a receptor antagonist in the bath allows us to exclude a possible diffusion of endothelin from the lumen to the bath. A luminal action of ET-3, implying ET_B receptors, has recently been demonstrated in proximal tubules while the agonist action on the basolateral side was associated with ET_C receptors (Knotek *et al.* 1996). On the other hand, the present study strongly suggests that the same receptor subtype, namely ET_B, is present on both sides of the epithelium. Moreover, since luminal and basolateral endothelin effects are not additive, it is probable that they are both mediated via similar transduction pathways. However, differences in signal transduction located at a step proximal to kinase activation remain to be elucidated.

In conclusion, the present work indicates that ET-1 and ET-3 exert an inhibitory action on MTAL and CTAL function after either basolateral or luminal addition. This effect is linked to ET_B receptors, unrelated to cAMP pathways, and mediated by a protein kinase C activation independent of increases in intracellular calcium. The effect might, in part, contribute to the potentiation of the natriuretic and diuretic action of the hormone on the collecting duct. However, the respective roles of ET-1 and ET-3 in physiological and pathological conditions remain to be elaborated.

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