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# The effect of big endothelin-1 in the proximal tubule of the rat kidney

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**1** An obligatory step in the biosynthesis of endothelin-1 (ET-1) is the conversion of its inactive precursor, big ET-1, into the mature form by the action of specific, phosphoramidon-sensitive, endothelin converting enzyme(s) (ECE). Disparate effects of big ET-1 and ET-1 on renal tubule function suggest that big ET-1 might directly influence renal tubule function. Therefore, the role of the enzymatic conversion of big ET-1 into ET-1 in eliciting the functional response (generation of 1,2-diacylglycerol) to big ET-1 was studied in the rat proximal tubules.

**2** In renal cortical slices incubated with big ET-1, pretreatment with phosphoramidon (an ECE inhibitor) reduced tissue immunoreactive ET-1 to a level similar to that of cortical tissue not exposed to big ET-1. This confirms the presence and effectiveness of ECE inhibition by phosphoramidon.

**3** In freshly isolated proximal tubule cells, big ET-1 stimulated the generation of 1,2-diacylglycerol (DAG) in a time- and dose-dependent manner. Neither phosphoramidon nor chymostatin, a chymase inhibitor, influenced the generation of DAG evoked by big ET-1.

**4** Big ET-1-dependent synthesis of DAG was found in the brush-border membrane. It was unaffected by BQ123, an $ET_A$ receptor antagonist, but was blocked by bosentan, an $ET_{A,B}$-nonselective endothelin receptor antagonist.

**5** These results suggest that the proximal tubule is a site for the direct effect of big ET-1 in the rat kidney. The effect of big ET-1 is confined to the brush-border membrane of the proximal tubule, which may be the site of big ET-1-sensitive receptors.

**Keywords:** Big endothelin-1; endothelin-1; endothelin-converting enzyme; phosphoramidon; chymostatin; 1,2-diacylglycerol; proximal tubule; brush-border membrane; basolateral membrane

## Introduction

Endothelin-1 (ET-1), a member of the endothelin family of regulatory peptides (Inoue *et al*., 1989), markedly influences renal vascular and tubular function (Simonson, 1993). A necessary step in the biosynthesis of ET-1 is the conversion of its precursor, big ET-1 (Yanagisawa *et al*., 1988), by the action of specific, phosphoramidon-sensitive, endothelin converting enzyme(s) (ECE), which have been recently cloned (Shimada *et al*., 1994; Xu *et al*., 1994). In the rat lung, the extracellular conversion of big ET-1 into ET-1 is sensitive to chymostatin, a chymotrypsin-like enzyme inhibitor (Hisaki *et al*., 1994), indicating the existence of additional enzymes participating in the conversion of big ET-1. It is possible that big ET-1 might directly influence renal tubule function in the rat since different tubule effects of big ET-1 and ET-1 have been observed in doses that produced similar systemic and renal vascular effects (Pollock & Opgenorth, 1994). Big ET-1, but not ET-1, produced an increase in sodium and water excretion (Pollock & Opgenorth, 1994). In addition the diuretic-natriuretic action of big ET-1 was only partially sensitive to phosphoramidon, although renal vascular responses to big ET-1 were completely abolished (Hoffman *et al*., 1994).

There are three subtypes of ET receptors which differ in their affinity toward endothelin peptides. The $ET_A$ receptor binds ET-1$\approx$ET-2$>>$ET-3, the $ET_B$ receptor binds all endothelins with equal affinity, while the $ET_C$ receptor has a high affinity for ET-3 (Masaki *et al*., 1994). Neither the $ET_A$ nor the $ET_B$ receptor bind big ET-1 (Clozel *et al*., 1993). Both $ET_A$ and $ET_B$ receptor activation results in the stimulation of phospholipase C (PLC) with subsequent generation of 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($IP_3$) (Aramori & Nakanishi, 1992).

Renal proximal tubular (PT) cells secrete ET-1 (Chen *et al*., 1993; Ong *et al*., 1995) and phosphoramidon-sensitive enzymatic activity which converts big ET-1 has been demonstrated in both human PT cells (Ong *et al*., 1995) and in LLC-$PK_1$ cells, a cell line of proximal tubule origin (Takada *et al*., 1992). The presence of endothelin receptors in the rat PT was shown by binding ($ET_B$) (Kohzuki *et al*., 1989; Uchida *et al*., 1993) and functional (Garcia & Garvin, 1994) studies. In our previous study, we suggested that endothelin-mediated stimulation of PLC occurs through the activation of $ET_B$ receptors in the brush-border membrane (BBM) and $ET_C$ receptors in the basolateral membrane (BLM) of the rat PT cells (Knotek *et al*., 1996).

In the present study the relationship between the functional response to big ET-1 (DAG formation) and its tissue conversion to ET-1 in the renal cortex was assessed. Our results suggest that big ET-1-dependent DAG production in the rat PT cells might reflect direct activation of endothelin receptors, probably located in the BBM, which are sensitive to bosentan (an $ET_{A,B}$ antagonist (Clozel *et al*., 1994)), but not to BQ123 (an $ET_A$ antagonist (Ihara *et al*., 1991)).

## Methods

### *Animals*

Studies were performed on male Wistar rats aged 3–4 months from the Departmental colony. The animals were killed by cervical dislocation, the kidneys were then removed, stripped

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of the perirenal fat and decapsulated. With a Stadie-Riggs microtome, sagittal cortical slices (wet weight 100 mg) were cut from each side of the kidney and immediately placed in ice cold PBS.

### *Isolation of proximal tubule cells and incubation with big ET-1*

PT cells were isolated from cortical tissue by collagenase digestion and the Percoll gradient method, as described originally by Gesek *et al.* (1987) and by Knotek *et al.* (1996). In the PT fraction, in the presence of 5 mM IBMX, a 3 fold increase in the level of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) was achieved after 20 min incubation with $10^{-7}$ M parathyroid hormone (PTH), whereas arginine vasopressin (AVP, 1 u $ml^{-1}$) was ineffective (data not shown). The enrichment of ratios of activities $mg^{-1}$ protein of alkaline phosphatase (AP, EC 3.1.3.1, proximal tubule marker) to hexokinase (H, EC 2.7.1.1., distal tubule marker) between the proximal tubule fraction and the cortical cell suspension ($AP/H_{proximal}/AP/H_{cortical}$) was about 4.5. Repeated microscopic examination confirmed the absence of glomeruli in the isolated cell suspension. Four out of six samples of isolated PT cells examined were negative and two were weakly positive for Von Willebrandt factor (ELISA, Boehringer), indicating a negligible contamination of isolated cells by endothelial cells. All incubations were carried out in 0.25 ml of Hank's solution (containing the following in mM: NaCl 135, KCl 5, $KH_2PO_4$ 0.44, $K_2HPO_4$ 0.36, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10, alanine 5 and HEPES 10; pH = 7.4) at 37°C in a thermostated shaker with continuous oxygenation. For experiments, cells were resuspended in Hank's solution (1–2 mg prot. $ml^{-1}$) and kept on ice. A dose-response curve for big ET-1 was obtained in the concentration range of $10^{-8}$–$10^{-6}$ M for 10 min. To obtain a time-course, the cells were exposed to big ET-1 ($10^{-7}$ M) for 0 and 45 s, and 2, 5, 10 and 15 min. In experiments with endothelin receptor antagonists and enzyme inhibitors, incubations were for 20 min. When used, bosentan, an $ET_{A,B}$ antagonist ($10^{-5}$ M) (Clozel *et al.*, 1994), BQ123, an $ET_A$ antagonist ($10^{-6}$ M) (Ihara *et al.*, 1991), phosphoramidon, an ECE inhibitor ($10^{-4}$ M) (Hisaki *et al.*, 1994) and chymostatin, a chymase inhibitor ($5 \times 10^{-5}$ M) (Hisaki *et al.*, 1994) were added 10 min before the addition of big ET-1 ($10^{-7}$ M, for 10 min) and were present throughout the incubation. Control PT cells were incubated for 20 min in the absence of inhibitors. Incubations were stopped by rapid freezing in liquid nitrogen. Cell viability was assessed by trypan blue exclusion before freezing (>90% at the end of experiments).

### *Incubation of cortical slices with big ET-1 and isolation of plasma membranes*

For all incubations two cortical slices (one sample) were placed into 3 ml Hank's solution. Incubations were carried out at 37°C in a shaking bath and oxygenated continuously. For determination of the membrane site of big ET-1 signalling, cortical slices were incubated with $10^{-7}$ M big ET-1 for 7 min. Incubations were terminated by adding the ice-cold PBS. BBMs were isolated by the Mg/EGTA-precipitation method (Biber *et al.*, 1981). Compared to the homogenate, the isolated BBMs were enriched in brush-border marker enzymes; 13 fold for leucine aminopeptidase (E.C. 3.4.11.2) and 8 fold for alkaline phosphatase (E.C. 3.1.3.1). The enrichment for $Na^+$-$K^+$-ATPase (E.C. 3.6.1.3), a BLM marker, was in the isolated BBMs <1, compared to homogenate. The BLMs were isolated from the kidney cortex by use of a Percoll gradient method as described by Scalera *et al.* (1981). Compared to homogenate, BLMs were enriched in $Na^+$-$K^+$-ATPase activity approx. 10 fold, while the enrichment for the leucine arylamidase and alkaline phosphatase was about 1. In both BBMs and BLMs low levels of the Von Willebrandt factor were detected in only one out of three samples examined, indicating a very slight contamination by endothelial cell membranes.

### *Mass assay of 1,2 diacylglycerol*

DAG from the plasma membranes or PT cells was extracted with 0.75 ml chloroform/methanol (1:2). Further extraction was performed as described by Folch *et al.* (1957). For the DAG measurement, a lipid extract was dissolved in 0.5 ml of chloroform and loaded on a silicic acid column (0.5 ml made in a Pasteur pipette), eluted with 1 ml of chloroform, dried and the mass measurement for DAG was then performed as described below. DAG kinase purification was achieved in a single step from rat brain by using DEAE-Sepharose column as described by Divecha & Irvine (1990). Mass measurement for DAG was performed in the following manner. Dried lipid was dissolved by the addition of 20 ml of CHAPS (9.2 mg $ml^{-1}$) and sonicated at room temperature for 15 s. After addition of 80 ml of buffer (50 mM Tris-acetate, 80 mM KCl, 10 mM magnesium acetate, 2 mM EGTA, pH 7.4), the reaction was started by the addition of 20 ml of DAG kinase enzyme followed by 80 ml of buffer containing 5 mM ATP and 1 mCi [$^{32}$P]-ATP. After 1 h at room temperature, the reaction was stopped by adding 750 ml of chloroform/methanol/HCl (80:160:1). Phosphatidic acid was extracted as described by Folch *et al.* (1957) and chromatographed on oxalate (1%)-sprayed thin layer chromatography (t.l.c.) plates with the following solvent system: chloroform/methanol/ concentrated ammonia/water (45:35:2:8). After autoradiography, the spots corresponding to phosphatidic acid were scraped off and their $^{32}$P content was determined by liquid scintillation counting. The DAG content in each sample was adjusted to be between 5 and 50 pmol, since the sensitivity of the assay was the highest in this range (Divecha *et al.*, 1991).

### *Measurements of ET-1 concentration*

ET-1 concentrations were determined in cortical slices unexposed to big ET-1, and after 10 min incubation with big ET-1 ($10^{-7}$ M). Before the addition of big ET-1, the slices were incubated for 10 min in Hanks' solution without, or with $10^{-4}$ M phosphoramidon, so that the total of each incubation was 20 min. After incubation the Hanks' solution was removed and the slices were washed three times in ice-cold PBS. ET-1 was extracted from the cortical tissue according to a previously published protocol (Fujita *et al.*, 1994). Briefly, the slices were homogenized for 2 min in 3 ml of ice-cold chloroform:methanol (2:1) containing 1 mM N-ethyl-maleimide (NEM). The homogenate was left overnight at 4°C and then 0.4 ml distilled water was added. The homogenate was centrifuged at 3,000 r.p.m. for 30 min and the supernatant was stored at −20°C until the analysis was performed. Extraction of ET-1 from the samples by use of Amprep C2 columns and measurement of its concentrations with the commercial ET-1-ELISA system (Amersham) were performed according to the manufacturer's instructions.

### *Materials*

Rat big ET-1 (1–39), phosphoramidon, chymostatin, parathyroid hormone (PTH), arginine vasopressin (AVP), isobuthylmethylxanthine (IBMX) and collagenase (type V) were from Sigma. BQ123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)) and bosentan were gifts from Dr Martine Clozel (F. Hoffmann-La Roche, Basel, Switzerland). Percoll was from Pharmacia. Pyruvate kinase, lactate-dehydrogenase and phosphoenolpyruvate were from Boehringer. [$^{32}$P]-adenosine 5′-triphosphate (ATP) (3000 Ci $mmol^{-1}$) was either from Amersham or ICN. All other chemicals used were of p.a. grade purchased commercially.

### *Statistical evaluation*

The data are shown as means ± s.e.mean of at least three measurements made in duplicate. For statistical analyses, Student's *t* test for unpaired samples was used. $P < 0.05$ was considered statistically significant.

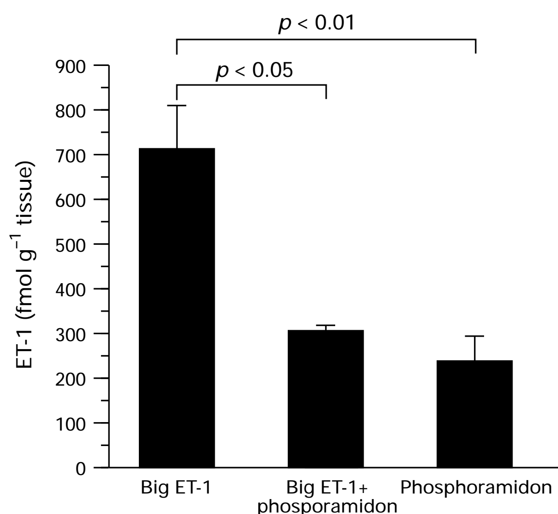


**Figure 1** ET-1 concentration in renal cortical tissue. Cortical slices were incubated with big ET-1 [$10^{-7}$ M] for 10 min. Where indicated phosphoramidon ($10^{-4}$ M) was added to slices 10 min before big ET-1. Each data point represents the mean ± s.e.mean from at least 4 experiments in duplicate. All other details are as described in the Methods section.

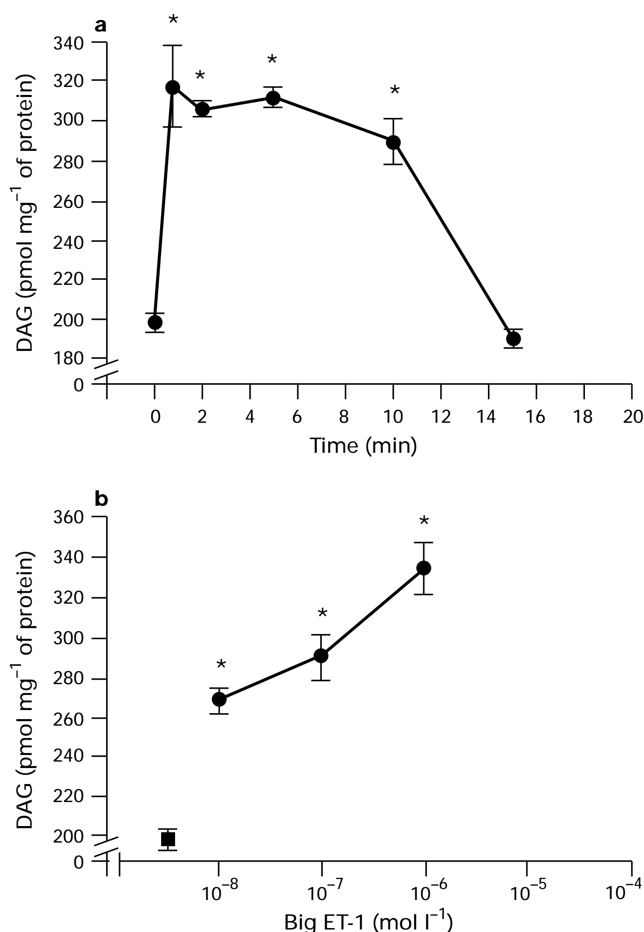


**Figure 2** Big ET-1-dependent 1,2-diacylglycerol (DAG) production in proximal tubular (PT) cells. (a) Time-course of DAG production in response to $10^{-7}$ M big ET-1. (b) Dose-dependence of DAG production in response to 10 min incubation with big ET-1 (●); control (■). Each data point represents the mean from at least 3 experiments in duplicate; vertical lines show s.e.mean. Asterisks indicate significant difference (*$P<0.01$, Student's $t$ test) from the control. All other details are as described in the Methods section.

## Results

In the first set of experiments the presence of phosphoramidon-sensitive ECE activity in the rat renal cortex was tested. We examined whether the pretreatment of cortical slices with phosphoramidon (an ECE inhibitor (Shimada *et al.*, 1994; Xu *et al.*, 1994)) attenuates ET-1 content in the cortical tissue following exposure of slices to $10^{-6}$ M big ET-1 for 10 min. As shown in Figure 1, in slices incubated with big ET-1 in the absence of phosphoramidon, ET-1 content was 2.3 times higher than in the presence of phosphoramidon. In addition, there was no significant difference between the ET-1 level in slices pretreated with phosphoramidon and subsequently incubated with big ET-1 and that in phosphoramidon-pretreated slices not incubated with big ET-1.

In freshly isolated PT cells, big ET-1 dose-dependently ($10^{-8}$–$10^{-6}$ M) produced an increase in DAG concentration (Figure 2). The increment in DAG was very rapid, reaching a plateau after 45 s of incubation of PT cells with $10^{-7}$ M big ET-1.

To test the functional role of the enzymatic conversion of big ET-1, PT cells were preincubated with $10^{-4}$ M phosphoramidon, or $5\times10^{-5}$ M chymostatin (chymase inhibitor (Hisaki *et al.*, 1994)) for 10 min. Neither the basal DAG level ($198\pm6$/ $194\pm9$/ $175\pm11$ pmol mg$^{-1}$ prot.; control/chymostatin/ phosphoramidon), nor the big ET-1-induced increase in DAG concentration were significantly influenced by these inhibitors (Figure 3).

Figure 4 shows the effect of the endothelin receptor antagonists on big ET-1-induced DAG generation. The response to big ET-1 was totally abolished when cells were pretreated with bosentan ($10^{-5}$ M), an $ET_{A,B}$-nonselective antagonist (Clozel *et al.*, 1994). Bosentan alone did not significantly affect the basal DAG level ($198\pm6$/ $180\pm8$ pmol mg$^{-1}$ prot.; control/bosentan). BQ123, an antagonist of $ET_A$ receptors (Ihara *et al.*, 1991), neither increased the basal DAG level ($198\pm6$/ $209\pm12$ pmol mg$^{-1}$ prot.; control/BQ123) nor prevented the response to big ET-1.

Finally, to determine the membrane site of big ET-1-dependent DAG formation, we measured DAG in BBM and

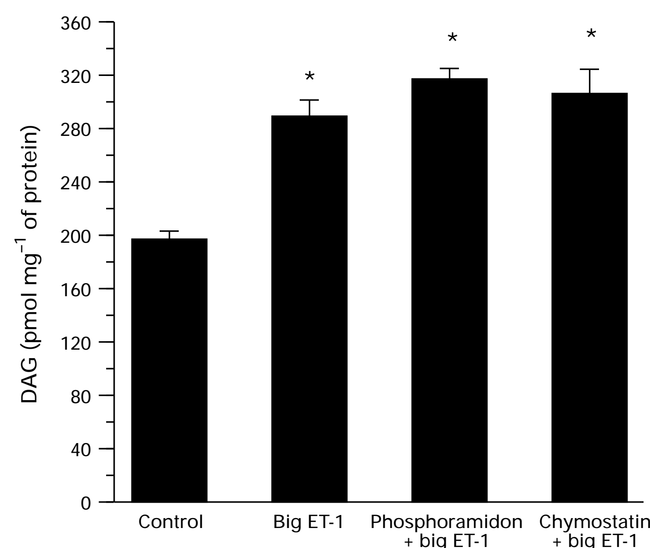


**Figure 3** The effect of phosphoramidon and chymostatin on big ET-1-induced 1,2-diacylglycerol (DAG) production in proximal tubular (PT) cells. When indicated, phosphoramidon ($10^{-4}$ M) or chymostatin ($5\times10^{-5}$ M) were added to cells 10 min before big ET-1 ($10^{-7}$ M for 10 min). Each data point represents the mean ± s.e.mean from at least 4 experiments in duplicate. Asterisks indicate significant difference (*$P<0.01$, Student's $t$ test) from the control. All other details are as described in the Methods section.

BLM separately, following a challenge of renal cortices with $10^{-7}$ M big ET-1. As shown in Figure 5, the increase in DAG was confined to the BBM.

## Discussion

$ET_A$ and $ET_B$ (Masaki *et al*., 1994), as well as $ET_C$ (Knotek *et al*., 1996) receptor activation results in the stimulation of PLC with subsequent generation of DAG. In the present study, the generation of DAG, after challenging the PT cells with big ET-1, was used as a tool to assess the functional response of PT cells to the big ET-1 with respect to its tissue conversion into ET-1. It was previously believed that only the converted form, ET-1, is functionally active (Yanagisawa *et al*., 1988). Functionally relevant conversion of big ET-1 into ET-1 is due to the action of ECE isozymes, some of which have been recently cloned (Shimada *et al*., 1994; Xu *et al*., 1994). A phosphoramidon-sensitive ECE activity has been demonstrated in LLC-$PK_1$ cells (Takada *et al*., 1992), and in human cultured PT cells (Ong *et al*., 1995). A novel chymostatin-sensitive enzyme activity capable of extracellular conversion of big ET-1 into ET-1 in the rat lung was demonstrated by Hisaki *et al*. (1994). However, our results suggest the possibility of an independent action of big ET-1 in the rat PT. Phosphoramidon, an effective inhibitor of ECE (Shimada *et al*., 1994; Xu *et al*., 1994), did not influence the generation of DAG by big ET-1 in isolated PT cells in our study. The inability of the chymase inhibitor, chymostatin (Hisaki *et al*., 1994), to prevent DAG accumulation in response to big ET-1 in our study, indicates that chymase does not mediate an alternative pathway for big ET-1 conversion to ET-1 in the PT. The amount of ET-1 present in the tissue following exposure to big ET-1 is thought to be a measure of the functional conversion of big ET-1 (Hisaki *et al*., 1994; Fujita *et al*., 1994). Accordingly, phosphoramidon abolished the accumulation of ET-1 in lung and renal tissue following perfusion with big ET-1 (Hisaki *et al*., 1994; Fujita *et al*., 1994). In the present study, inhibition of ECE was demonstrated by the reduction of tissue ET-1 content, in cortical slices pretreated with phosphoramidon and incubated subsequently with big ET-1, to the level observed in slices not exposed to big ET-1. During an equivalent incubation period phosphoramidon did not affect the basal DAG level in PT cells, excluding the possibility of a nonspecific effect of phosphoramidon on PLC. Thus, signalling by big ET-1 and its conversion into ET-1 were clearly distinguished by the use of phosphoramidon. Such ECE-independent action of big ET-1 is in agreement with the findings of Hoffman *et al*. (1994); they noticed that, unlike the vascular effects of big ET-1, diuretic and natriuretic effects of big ET-1 in the rat were only partially sensitive to phosphoramidon. In addition, following jugular intravenous infusion, ET-1 did not influence renal excretion of sodium and water, whilst big ET-1 evoked a strong natriuresis and diuresis, despite quantitatively similar renal and systemic vascular responses evoked by both agents (Pollock & Opgenorth, 1994). Our results suggest that this can be best explained by a direct tubular action of big ET-1.

There are three subtypes of the endothelin receptor ($ET_A$, $ET_B$ and $ET_C$) which differ in their affinity toward endothelin isoform peptides (Masaki *et al*., 1994). There is some controversy regarding the presence of endothelin receptors in the rat PT. Endothelin receptors in proximal tubules of the rat have been demonstrated by binding ($ET_B$) (Kohzuki *et al*., 1989; Uchida *et al*., 1993), as well as by functional studies (Garcia & Garvin, 1994). However, Terada *et al*. (1992) failed to detect $ET_B$ and $ET_A$ receptor mRNAs in the rat PT. This may be due either to low levels of endothelin receptor mRNAs expression or to the presence of novel endothelin receptor subtypes. In the study of Dean *et al*. (1994), after *in vivo* application of [$^{125}$I]-ET-1 no binding to the rat PT was observed. Although we cannot offer an explanation for the latter results concerning the PT, it is interesting to note that Dean *et al*. (1994) also failed to demonstrate endothelin binding to the mesangial cells and to the medullary collecting ducts, although these structures are clearly recognized as sites of ET-1 receptors in rat kidney (Simonson, 1993). Using the same approach as in the present study, we have recently demonstrated $ET_B$-mediated stimulation of PLC in the BBM and stimulation of PLC by an ET-3-selective mechanism (probably $ET_C$-mediated) in the BLM of rat PT (Knotek *et al*., 1996). In the present study, the increase in DAG in response to big ET-1 was completely blocked by bosentan, an $ET_{A,B}$-nonselective antagonist (Clozel *et al*., 1994), while BQ123, an $ET_A$ antagonist

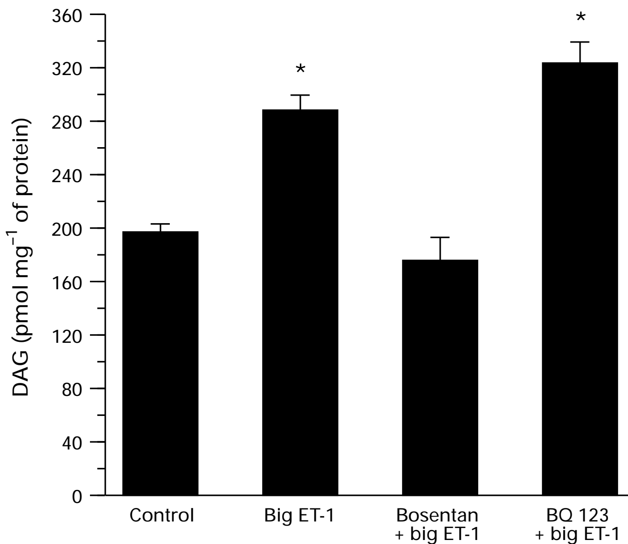


**Figure 4** Effect of the endothelin receptor antagonist on big ET-1-induced 1,2-diacylglycerol (DAG) production in proximal tubular (PT) cells. PT cells were incubated with big ET-1 [$10^{-7}$ M] for 10 min. Bosentan ($10^{-5}$ M) and BQ123 ($10^{-6}$ M) were added 10 min before big ET-1. Each data point represents the mean$\pm$s.e.mean from at least 4 experiments in duplicate. Asterisks indicate significant difference (*$P<0.01$, Student's *t* test) from the control. All other details are as described in the Methods section.

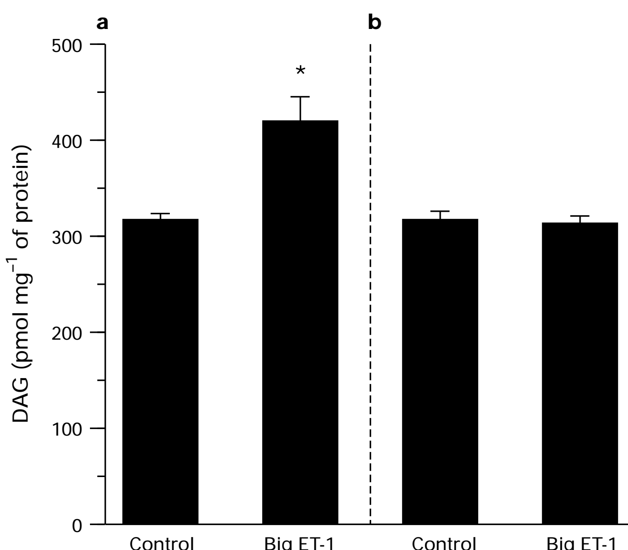


**Figure 5** Membrane site for big ET-1-dependent 1,2-diacylglycerol (DAG) formation. (a) Brush-border membranes (BBM) and (b) basolateral membrane (BLM) were isolated from cortical slices incubated with big ET-1 ($10^{-7}$ M for 7 min). Each data point represents the mean$\pm$s.e.mean from at least 3 experiments in duplicate. Asterisks indicate significant difference (*$P<0.01$, Student's *t* test) from the control. All other details are as described in the Methods section.

(Ihara *et al*., 1991), had no effect on big ET-1-dependent DAG production. This excludes the possibility of contamination by mesangial cells, since they posses $ET_A$ receptors (Simonson, 1993). Our results correspond with those from the study of Pollock & Opgenorth (1994), where tubular effects of big ET-1 were also insensitive to BQ123. The endothelin receptor subtype involved in the action of big ET-1 noted in the present study cannot be currently assigned. Neither $ET_A$ nor $ET_B$ bind big ET-1 (Clozel *et al*., 1993). The presence of a novel, ET-1-sensitive, endothelin receptor subtype in the dog PT was recently suggested (Clavell *et al*., 1995). According to our data, a possible, additional but novel big ET-1-sensitive endothelin receptor subtype exists in the rat PT which may be similar to the $ET_B$ subtype. Further studies are needed to clarify this. Because ET-1 evoked DAG formation only in BBM, this membrane domain of PT cell may be the site of the big ET-1-activated receptors (Banfić *et al*., 1993).

The physiological significance of a direct action of big ET-1 on the PT is not clear. The PT is the site of ET-1 production (Chen *et al*., 1993; Ong *et al*., 1995) and ET-1 could regulate $Na^+/H^+$-exchange and $Na^+/HCO_3^-$-cotransport activities (Garcia & Garvin, 1994), as well as participate in PT growth responses (e.g. regeneration after ischaemic injury) (Ong *et al*., 1995). Since PT cells in culture secrete ET-1 across the BLM (Ong *et al*., 1995), an autocrine action of big ET-1 in PT appears unlikely. Because of its size, big ET-1 may not be freely filtered unless the permeability of the glomerular filtration membrane is increased (Arendshorst & Navar, 1988). Since big ET-1, unlike ET-1, does not signal in hepatic slices (Beara-Lasić *et al*., unpublished observation), big ET-1 may only influence renal tubular function, perhaps acting in an endocrine manner, in states when the permeability of the glomerular membrane is increased. A possible role for big ET-1 in the conditions associated with glomerular proteinuria deserves further investigation.

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