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Endothelin Increases Intracellular Free Calcium in Isolated Rat Nephron

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In the freshly isolated rat nephron, the effect of endothelin-1, -2 and -3 (ET-1, -2 and -3) on cytosolic free calcium concentration ($[Ca^{2+}]_i$) was determined using the fluorescent indicator Fura-2/AM. $[Ca^{2+}]_i$ increase was investigated in 9 parts of the single nephron including glomerulus (Glm), S_1, S_2, S_3, cortical and medullary thick ascending limb and cortical (CCT) and outer medullary collecting tubule (OMCT). Endothelins increased $[Ca^{2+}]_i$ in Glm (ET-1; 127±17%, ET-2; 93±5%, ET-3; 169±17%), CCT (ET-1; 30±6%, ET-2; 38±19%, ET-3; 158±18%) and OMCT (ET-1; 197±11%, ET-2; 195±11%, ET-3; 215±37%) at 10^{-7} M. In OMCT, ET-1 and ET-2 increased $[Ca^{2+}]_i$ in a dose-dependent manner (10^{-10}~10^{-6} M). To the contrary, ET-3-induced $[Ca^{2+}]_i$ rise was begun from 10^{-12} M. BQ-123Na, an antagonist of ET_A receptor, at 10^{-4} M inhibited about 30% of $[Ca^{2+}]_i$ rise induced by ET-1 and -3. Binding experiments using [^{125}I]ET-3 showed the existence of ET_B receptor in OMCT. This binding was replaced by ET-1, ET-2 or ET-3 by the almost same degree but not by angiotensin II or vasopressin.

Key Words: Endothelin, Intracellular free calcium, Outer medullary collecting tubule

INTRODUCTION

A vasoactive peptide, endothelin is a potent vasoconstrictor peptide originally characterized from the culture supernatant of porcine aortic endothelial cells consisting of 21 amino acid residues containing two sets of intrachain disulfide linkages (Yanagisawa et al, 1988). Some investigators reported that ET was produced in not only vascular endothelial cells, but also kidney, neural cells and epithelial cells (Yanagisawa et al, 1988, Giaid et al, 1989, Yoshizawa et al, 1990, Yoshimi et al, 1989).

Analysis of a human genomic library has led to the identification of three isopeptides of ETs, designated ET-1, ET-2 and ET-3 (Yanagisawa et al, 1988, Inoue et al, 1989). In human endothelins, the structural differences of ET-2 and ET-3 from that of ET-1 were 2 and 6 amino acid residue sequences, respectively. And their genes appeared to be present in the rat, porcine and human genome. Animal experiments, mainly on rats, have shown that the kidney vasculature was one of the vascular beds most sensitive to endothelin (Masaki 1989, Tomobe et al, 1988). Endothelin induced water and sodium excretion in concentration not causing GFR decrease (King et al, 1989). In addition to its action on vascular cells, ET has been reported to bind to and act upon epithelial cells (Totsune et al, 1989). The peptide apparently inhibits Na^+-K^+-ATPase in isolated epithelial cells of the inner medullary collecting tubules of rabbit (Zeidel et al, 1989). And endothelin has been known to increase cytosolic free calcium in target cells, e.g. blood vessels, smooth muscle cells and renal epithelial cells, which is mediated by the activation of inositol triphosphate (Vogel et al, 1977, Sugiura et al, 1989, Ankorima-Stark et al, 1997, Marsden et al, 1989, Naruse et al, 1991).

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Many papers have been published on the cells exhibiting the ET_A and ET_B type receptors (Martin et al, 1990, Sakurai et al, 1990, Arai et al, 1990, Ogawa et al, 1991). Recently, Samson et al, (1990) reported existence of an isoform receptor in cultured anterior pituitary cells which acted stronger at ET-3 than ET-1. This receptor was called ET_C-type receptor which did not become clear.

In the present study, therefore, we examined the effects of ET-1, -2 and -3 on transient $[Ca^{2+}]_i$ and distribution of endothelins receptor subtypes in nephron segments freshly isolated from rat kidneys.

METHODS

Animals

Male Sprague-Dawley rats weighing 200~240 g were purchased from Saitama Experimental Laboratories (Saitama, Japan) and housed in commercial equipment in a conventional environment for at least 3 days prior to use. They were provided pelleted standard diet (Oriental Yeast, Osaka, Japan) and tap water *ad libitum.*

Materials

The materials used were purchased as following: Fura-2/AM from Dojin Laboratories (Japan); ET-1, -2, -3 and angiotensin II from Peptide Institute Inc. (Japan); [^{125}I] ET-3 from Amersham (Japan); aprotinin, bacitracin, N-ethylmaleimide, verapamil, 8-(N, N-diethyl-amino)octyl-3,4,5-trimethoxy-benzoate (TMB-8), vasopressin and collagenase from Sigma (USA); 8-hydroxyquinoline from Aldrich (USA); BQ-123Na was kindly donated by Dr. F. Ikemoto of Banyu Pharmaceutical Co., Ltd. (Japan). All other chemicals were of the highest grade available.

Preparation of nephron segments

Microdissection techniques of various nephron segments have been previously described (Jung et al, 1989). Briefly, male Sprague-Dawley rats were decapitated, and left kidneys were perfused with modified Hanks' solution containing (in mM): NaCl, 127; $MgSO_4$, 0.8: Na_2HPO_4, 0.33: KH_2PO_4, 0.44: $MgCl_2$, 1: CH_3COONa, 10; Trizma base, 10: $CaCl_2$, 1 (pH 7.4) containing 2 mM pyruvate, 2 mM aspartic acid, 2 mM glutamic acid, 0.1% (wt/vol) bovine serum albumin (BSA) and 0.1% (wt/vol) collagenase (type I, 240 U/mg). Perfused kidneys were removed, and cut with 1mm thickness on the corticomedullary axis. The collagenase treatment was carried out at 37°C for 20 min with same solution. Microdissection of nephron segments was carried out at 4°C with fine siliconized stainless steel needle. The length of each nephron segments were measured with an ocular micrometer. The nephron segments were identified as previously reported (Kriz & Bankir 1988). The early proximal tubule (S_1) was identified by its attachment to a glomerulus (Glm). The second portion of the proximal tubule (S_2) was the transitional part of convolution to the straight portion. The late proximal tubule (S_3) was identified by its attachment to the thin descending limb of Henle's loop. The medullary (MTAL) thick ascending limbs of Henle's loop was dissected from the inner stripe of the outer medulla. The distal tubule including connecting tubule was identified by its attachment to Glm. The cortical and medullary collecting tubules (CCT and OMCT) were dissected from the medullary ray of the cortex and the inner stripe of outer medulla, respectively.

The methods for $[Ca^{2+}]_i$ measurement have been previously described (Cha et al, 1995, Jung & Endou 1990, Grynkiewicz et al, 1985). Briefly, isolated nephron segments placed on the center of a serological glass were incubated at 25°C for 20 min with modified Hanks' solution containing 10 μM Fura-2/AM, 10 mM CH_3COONa, 2 mM pyruvate, 2 mM aspartic acid, 2 mM glutamic acid, 10% fetal bovine serum (FCS) and 0.5 mM $CaCl_2$ (pH 7.40) on a vibratile stage for histological immunostaining instruments. Fluorescence-loaded cells were washed three times with the same solution for exception of Fura-2/AM and FCS. The fluorescence loaded tubules together with 90 μl medium were transferred on a cover glass siliconized freshly, and the fluorescence of a nephron segment was measured at 25°C using a 2 wave-length microscopic fluorometer (CAM 220, JASCO, Japan). During monitoring fluorescence intensity at two wave length, agonists or antagonists (10 μl) were mixed with the 90 μl medium. In each nephron segment, the iris of fluorescence was maximally adjusted to each nephron diameter. Fluorescence emission at 510 nm was measured in response to excitation at 340 nm (F340) and 380 nm (F380),

and intracellular calcium concentrations were determined using the following formula:

$$[Ca^{2+}]_i = [(R\text{-}Rmin)/Rmax\text{-}R)] \times B \times 224$$

where R is the ratio F340/F380, Rmin is a ratio in the absence of calcium and Rmax is a ratio in the presence of an excess of calcium, and B is determined by the ratio of F380 in the absence of calcium to F380 in the presence of an excessive calcium.

Binding experiments of [^{125}I] ET-3 to nephron segments.

[^{125}I] ET-3 binding to OMCT was carried out principally by the method of Butlen et al, (1988) with a slight modification. Kidney slices were rinsed with dissection medium consisting of (in a final concentration in mM) NaCl, 137; KCl, 5; $MgSO_4$, 0.8; Na_2HPO_4, 0.33; NaH_2PO_4, 0.44; $NaHCO_3$, 4; $MgCl_2$, 1; glucose, 5; CH_3COONa, 10; $CaCl_2$, 1; HEPES-NaOH, 20 (pH 7.40); containing 2 mM pyruvate, 2 mM aspartic acid, 2 mM glutamic acid, 0.1% (wt/vol) bovine serum albumin (BSA) and 3% dextran (molecular weight 40,000) and nephron segments were dissected. The 5 mm of tubules were incubated for 1 hr at 25°C in a 3 μl droplet of microdissection medium complemented with 0.05% aprotinin, 0.1% bacitracin, 1 mM N-ethylmaleimide, 1 mM 8-hydroxyquinoline, and 200 pM of [^{125}I] ET-3 and with (nonspecific binding) or without (total binding) 10^{-7} M of unlabeled ET-1 or ET-3. The incubation was terminated by adding 200 μl of chilled microdissection medium. An 1 μl droplet containing the nephron segments was aspirated and washed twice with 200 μl of medium. Finally, the nephron segments in 1 μl droplet was transferred and radioactivity was counted using a gamma spectrometer (ARC-300, Aloka, Japan). Specific binding was calculated as the difference between total binding and nonspecific binding.

Statistics

All results are means±SE. Student's-*t* test was used for statistical comparisons between two groups.

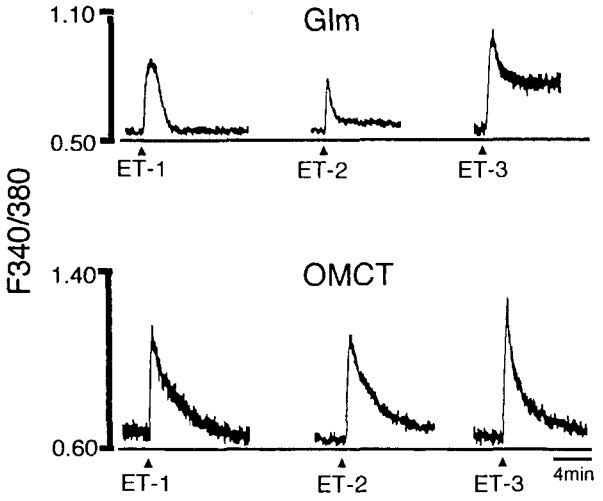

Fig. 1. Representative signalings of effects of ET-1, -2 and -3 on intracellular free calcium concentration in isolated rat Glm and OMCT. The concentration of ETs was 10^{-6} M.

RESULTS

To define the effects of endothelins on intracellular free calcium in isolated rat nephron segments, we utilized calcium-sensitive fluorescent indicator, Fura-

Table 1. Effects of ET-1, -2 and -3 on $[Ca^{2+}]_i$ of isolated rat single-nephron segments

	increasing% of basal $[Ca^{2+}]_i$			
	Glm	S_1	S_2	S_3
ET-1	127±17.2**	N.C.	N.C.	N.C.
ET-2	93±17.2**	N.C.	N.C.	N.C.
ET-3	169±17.2**	N.C.	N.C.	N.C.
	MTAL	DCT	CCT	OMCT
ET-1	N.C.	N.C.	30±6*	197±11**
ET-2	N.C.	N.C.	38±19*	195±11**
ET-3	N.C.	N.C.	158±18**	215±36**

Values are means±SE. of 4 experiments. ET, endothelin; Glm, glomerulus; S_1, early proximal tubule; S_2, transitional part of convolution to the straight portion; S_3, late proximal tubule; MTAL, medullary portion of thick ascending limb of Henle; DCT, distal convoluted tubule; CCT, cortical collecting tubule; OMCT, outer medullary collecting tubule. *:$p<0.05$, **:$p<0.01$ vs. resting $[Ca^{2+}]_i$.

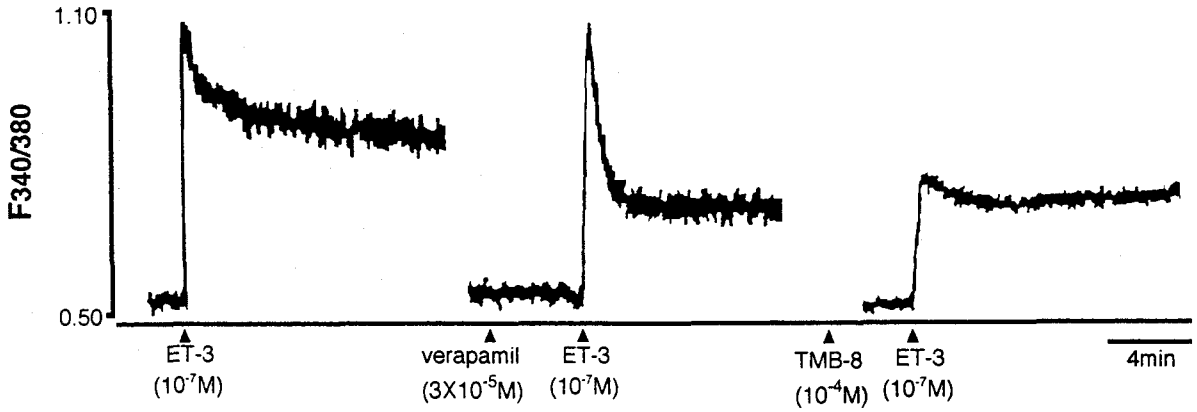

Fig. 2. Representative signalings of effects of verapamil or TMB-8 on ET-3-induced $[Ca^{2+}]_i$ transients in isolated rat Glm. Verapamil or TMB-8 were pretreated for 4 min or TMB-8 for 30 min, respctively.

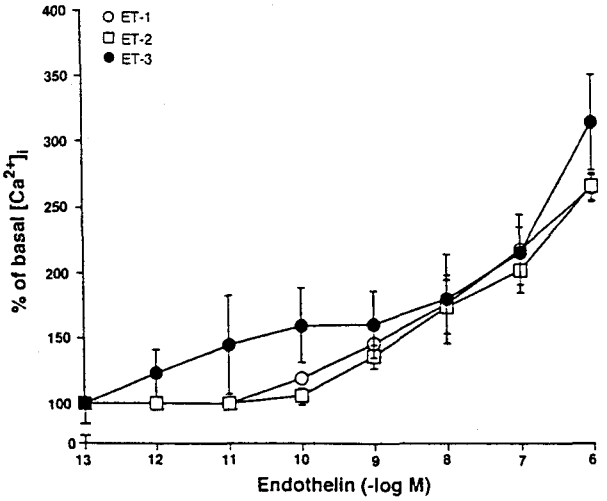

Fig. 3. Dose-response curves of ET-1, -2 and -3 on $[Ca^{2+}]_i$ transients in isolated rat OMCT. Highest $[Ca^{2+}]_i$ peaks of each responses were calculated vs. basal $[Ca^{2+}]_i$. Values were presented mean ± SE. of 6~9 experiments.

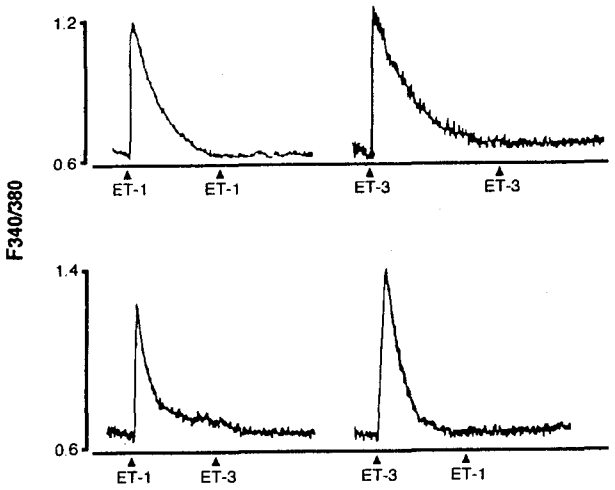

Fig. 4. Representative signalings of repeated effect and cross desensitization of ET-1 and/or ET-3. The concentration of endothelins was 10^{-6} M.

2/AM. Fig. 1 shows typical signalings of ET-1-, ET-2- and ET-3-induced $[Ca^{2+}]_i$ in Glm and OMCT. The patterns of changes in OMCT by ET-1, -2 and -3 (10^{-7} M) in fura-2 fluorescence were similar in other segments responded. ETs increased fluorescence intensity rapidly to a peak within 20 sec, followed by a fall to a steady level within 3~10 minutes.

From the fluorescence changes responsible to ET -1, -2 and -3, the responsiveness to endothelins along the nephron of rats is summarized in Table 1. The order of transient increase by ET-1, ET-2 and ET-3 were OMCT > Glm > CCT. The other segments had no responsiveness to endothelins.

In order to assess a possible source of endothelin-induced $[Ca^{2+}]_i$ increase, Glm was tested. Characteristically typical tracings peak in Glm show ET-3-induced $[Ca^{2+}]_i$ to be biphasic, a rapid transient peak and a long sustained phase (Fig. 2). 3×10^{-5}M of verapamil, a calcium channel blocker, and 10^{-4}M of TMB-8, a calcium release inhibitor from intracellular calcium pool, were utilized for ET-3-induced $[Ca^{2+}]_i$. In presence of verapamil, a rapid transient phase did not change but a sustained phase was inhibited slightly. And a rapid transient phase was inhibited by the pretreatment of TMB-8 for 30 min. Similar results

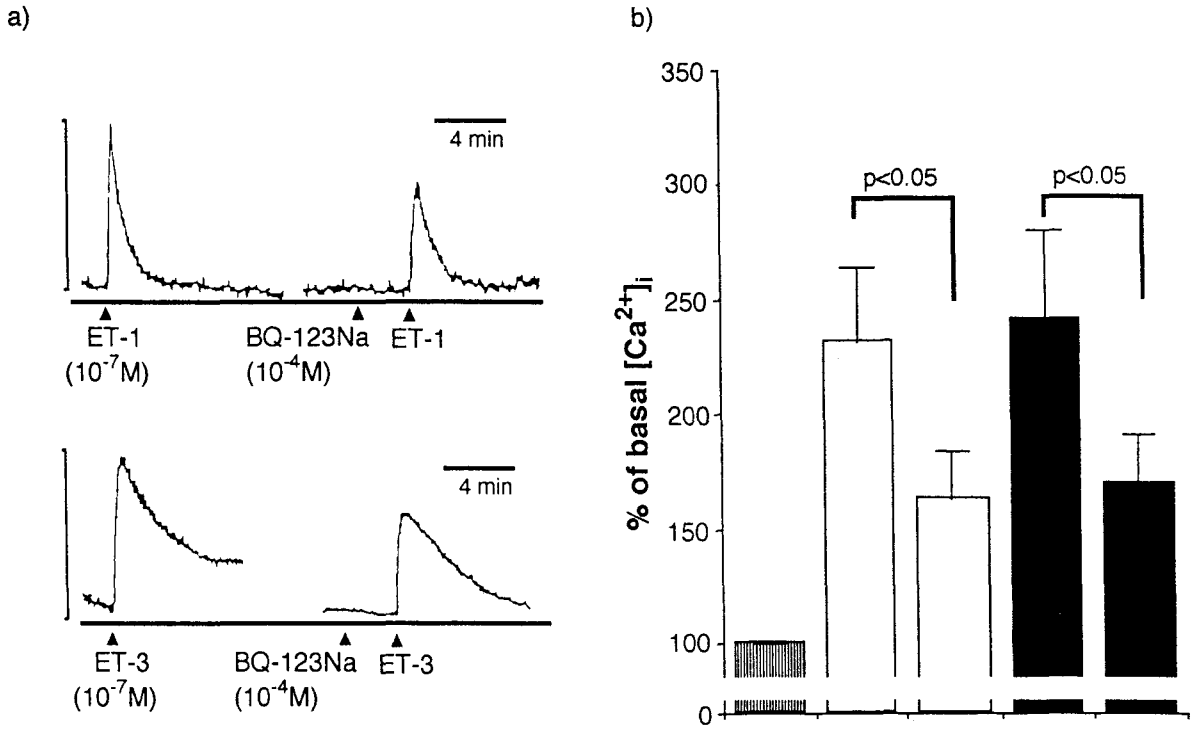

Fig. 5. Antagonistic effect of BQ-123Na in isolated rat OMCT.
a) Representative signalings of effect of BQ-123Na on ET-1- or ET-3-induced $[Ca^{2+}]_i$.
b) Summarization of effect of BQ-123Na on ET-1- or ET-3-induced $[Ca^{2+}]_i$.
Values are expressed mean ± SE. of 5 rats.

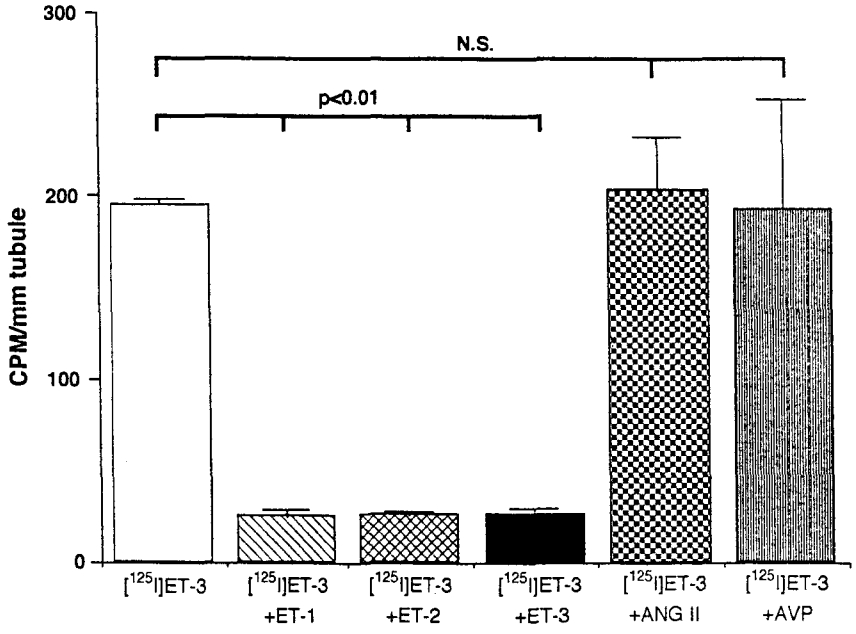

Fig. 6. Competitive inhibitory effects of ET-1, -2, -3, ANG II and AVP in isolated rat OMCT. Binding experiment was performed with unlabelled agonists (10^{-7} M) and [^{125}I] ET-3 (200 pM). Values were expressed mean ± SE (N=4).

obtained in OMCT, too (data were not shown).

Fig. 3 depicts the dose-response of endothelins in isolated rat OMCT. Each $[Ca^{2+}]_i$ peak vs. basal $[Ca^{2+}]_i$ was calculated. ET-1 and -2 increased $[Ca^{2+}]_i$ in a dose-dependent manner from 10^{-10} to 10^{-6} M. On the other hand, ET-3-induced $[Ca^{2+}]_i$ increase began at 10^{-12} M.

Next we investigated repeated effects of ET-1 and -3 on $[Ca^{2+}]_i$ transients in OMCT, and typical tracings are demonstrated in Fig. 4. First treatment with 10^{-6} M ET-1 induced transiently $[Ca^{2+}]_i$ rise and the second treatment with the same concentration of ET-1 did not change the resting $[Ca^{2+}]_i$. In a combined experiments of ET-1 with -3, ET-1 or -3 inhibited the $[Ca^{2+}]_i$ subsequent increase by ET-3 or ET-1, respectively. Both agents evoked similar $[Ca^{2+}]_i$ transients by either alone.

To clarify the kinds of endothelin receptors in OMCT, we investigated antagonistic effects of BQ-123Na, which is ET_A receptor antagonist (IC_{50}:22 nM in aorta) relatively having a weak antagonistic effect to ET_B receptor (IC_{50}:18 μM in cerebellum). In isolated OMCT, Fig. 5 showed that 10^{-7} M ET-1- and ET-3-induced $[Ca^{2+}]_i$ were slightly inhibited but significant by the pretreatment with 10^{-4} M BQ-123Na for 3 min ($163.6 \pm 21.5\%$ to $231.7 \pm 32.7\%$ for ET-1 and 210.0 ± 22.0 to 314.0 ± 38.3 for ET-3).

In order to further characterize specific receptors for ET-3 in OMCT, radioligand binding experiments were performed using [^{125}I] ET-3. Nonspecific binding was defined by the coincubation with 10^{-7} M unlabeled ET-3. The binding study as well as $[Ca^{2+}]_i$ determination, was done at 25°C. Ligand binding reached a plateau at 60 min, specific bindings became greater with increasing concentrations of [^{125}I] ET-3 in the presence of excessive unlabeled ET-3 (10^{-7} M) (data not shown). Competitive inhibitory experiments were performed with 200 pM [^{125}I] ET-3 and 10^{-7} M unlabeled peptides such as ET-1, -2, -3, angiotensin II and vasopressin in OMCT (Fig. 6). Unlabeled ET-1, -2 and -3 at 10^{-7} M inhibited [^{125}I] ET-3 binding by 86.9, 86.7 and 86.2% ($p < 0.01$), respectively. The vasoconstrictive peptidyl hormones failed to inhibit the ET-3 bindings.

DISCUSSION

Endothelin-induced transient $[Ca^{2+}]_i$ increase in blood vessel, smooth muscle and renal epithelial cells has been reported previously (Vogel et al, 1997, Ankorina-Stark et al, 1997, Marsden et al, 1989, Naruse et al, 1991). Those investigations were carried out using ET-1 and ET-2. According to these studies, endothelin had most potent vasoconstrictive effect among the well-known vasoactive peptidyl agents. Endothelin-1 stimulated the metabolism of inositol phosphates and induced the mobilization of $[Ca^{2+}]_i$ in vascular smooth muscle, cultured rat medullary interstitial cell and mouse renal epithelial cells. The present findings clearly demonstrate target cells of endothelins within the kidney.

Endothelin-3 induced $[Ca^{2+}]_i$ transient in Glm showed biphasic, a rapid transient peak and a long sustained phase. Since usage of TMB-8 (Chiou et al, 1975) and verapamil (Karaki et al, 1991) partly attenuated the initial peak height of $[Ca^{2+}]_i$ transients of a rapid phase and a long sustained phase induced by ET-3 in isolated Glm and OMCT. These results strongly suggested that ET-3-induced transient $[Ca^{2+}]_i$ increase is mainly form intracellular calcium stores. The sustained phase of $[Ca^{2+}]_i$ transient occurs largely by Ca^{2+} influx through plasma membranes. Our data well agreed with many investigation, suggesting that endothelin at high concentrations may raise $[Ca^{2+}]_i$ through release of Ca^{2+} from intracellular stores, probably mediated by inositol 1,4,5-triphosphate production particularly (Badr et al, 1989, Mitsuhoshi et al, 1989) and may do so through increased Ca^{2+} influx across plasma membranes, which may or may not be sensitive to Ca^{2+}-channel blockers (Pang et al, 1989, Watanabe et al, 1989).

The ET-1 and -2 increased $[Ca^{2+}]_i$ in a dose-dependent manner 10^{-10} to 10^{-6} M. But ET-3-induced $[Ca^{2+}]_i$ rise began at 10^{-12} M to 10^{-6} M. And data from experiments of repetition and desensitization showed same tendency of ET-1- and -3-induced $[Ca^{2+}]_i$ in OMCT. BQ-123Na (Ihara et al, 1991) significantly inhibit ET-1- and ET-3-induced $[Ca^{2+}]_i$ increase in OMCT. These meant that distributed receptors in OMCT were ET_B-type receptors.

Sarafotoxins S6 b (SRTX) is a group of 21-residue isocardiotoxic peptides, isolated from the venom of the snake *Artactaspis engaddensis*, which is rich in cysteine and shows a high sequence homology with endothelin (Kloog et al, 1988, Sokolvsky 1991). In preliminary experiments, distribution of SRTX-induced $[Ca^{2+}]_i$ transients in isolated rat single

nephrons were similar to ET-1 (data were not shown).

Chizuko et al, (1989) reported autoradiographic distribution in rat tissues of binding sites for endothelin. According to their results, [^{125}I]endothelin binding sites are distributed in heart ventricle, kidney, lung, intestine, adrenal gland and brain. In kidney, radiolabelled ET-1 receptor in Glm, cortex, inner medulla and renal papilla (Davenport et al, 1989), but distribution of ET-3 binding site in kidney was obscure still now. Therefore, distribution of ET-3 binding was performed with measurement of $[Ca^{2+}]_i$. From [^{125}I]ET-3 binding and competitive inhibitory experiments, the receptor in OMCT was cleared to be ET_B.

In conclusion, ETs increase $[Ca^{2+}]_i$ in Glm, CCT and OMCT. According to the antagonist and binding experiments, endothelin receptors existing in renal epithelial cell were ET_B subtype.

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