Effects of Endothelin on Peptide-dependent Cyclic Adenosine Monophosphate Accumulation along the Nephron Segments of the Rat

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Abstract

We investigated the tubular action of endothelin in rat nephron segments. The effects of endothelin on arginine vasopressin (AVP)-, parathyroid hormone-, glucagon-, calcitonin-, and isoproterenol-dependent cAMP accumulation were studied. The following nephron segments were microdissected: glomerulus (GI), proximal convoluted tubule (PCT), cortical and medullary thick ascending limbs of Henle’s loop (cTAL and mTAL, respectively), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). Endothelin dose dependently (10^{-8}-10^{-10} M) inhibited AVP-dependent cAMP accumulation in GI, OMCD, and IMCD. This effect was independent of the presence or absence of phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, or indomethacin, but was abolished in the presence of protein kinase C inhibitor H-7. Protein kinase C stimulator diacytanol glycerol mimicked the effect of endothelin. On the other hand, endothelin had no inhibitory effect on AVP-dependent cAMP accumulation in cTAL or mTAL, parathyroid hormone-dependent cAMP accumulation in GI and PCT, or glucagon-, calcitonin-, and isoproterenol-dependent cAMP accumulation in OMCD.

We conclude that endothelin specifically inhibits AVP-dependent cAMP accumulation in CCD, OMCD, and IMCD through activating protein kinase C. This effect possibly has a role in maintaining urine volume to counteract the decrease in GFR caused by endothelin itself. (J. Clin. Invest. 1990. 85:2014-2018.) arginine vasopressin • protein kinase C • microdissection • collecting duct • diuresis

Introduction

Endothelin is a new peptide and has potent vasoconstrictor activity, mediated by Ca entry through a plasma membrane Ca channel (1). Immunoreactive endothelin was detected in plasma of humans (2), suggesting that endothelin has a potential role as a circulating hormone. In the isolated perfused rat kidney, endothelin decreased the GFR, while the same dose of angiotensin II had little effect on GFR (3). A recent report showed that the urinary flow rate increased after endothelin administration in the anesthetized rat, even though GFR and renal plasma flow significantly decreased, by ~ 50% (4). Another work indicated that endothelin decreased urinary sodium excretion, urine osmolality, and osmolar clearance, and that it increased free water clearance (5). Recent autoradiographic study indicated the presence of binding sites for endothelin in medulla as well as glomerulus of the rat kidney (6). These observations suggest that endothelin may have target sites in the kidney other than the glomerulus, possibly on renal tubules.

Three possibilities can be considered if endothelin has a diuretic action on renal tubules. The first possibility is the direct stimulation or inhibition of epithelial transport. A second is stimulation of other factors that have diuretic action on tubules, such as bradykinin, prostaglandins, and atrial natriuretic factor. A third is inhibition of the action of hormones that have antiuiteric action on tubules, such as vasopressin (AVP), catecholamines, and mineralocorticoids. AVP is the most important of the above mentioned factors. Therefore, our investigation is mainly focused on the interaction between AVP and endothelin in the distal segments where urine can be maximally concentrated. In addition, the effects of several peptides such as PTH, glucagon, calcitonin, and isoproterenol were also investigated to see the specificity and sites of the action of endothelin.

Methods

Endothelin, PTH, and salmon calcitonin were purchased from Peptide Institute, Osaka, Japan. Endothelin was dissolved with 0.001% Triton X-100. AVP, glucagon, isoproterenol, 3-isobutyl-1-methylxanthine (IBMX),1 indomethacin, 1,2-dioctanoyl-sn-glycerol, TCA, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). Protein kinase C inhibitor H-7, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine dihydrochloride was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Nicardipine was a kind gift from Yamanouchi Pharmaceutical Ltd. (Tokyo, Japan). All other chemicals were of first grade purity.

Solutions. The microdissection solution (solution 1) was a Hepes-buffered solution of the following concentration (in millimolar): 130 NaCl, 5 KCl, 1 NaH2PO4, 1 MgSO4, 1 Ca lactate, 2 Na acetate, 5.5 glucose, 5 l-alanine, 2 l-leucine, and 10 Heps, and 0.05% BSA was

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1. Abbreviations used in this paper: ANP, atrial natriuretic peptide; CCD, cortical collecting duct; cTAL, cortical thick ascending limb of Henle’s loop; GI, glomerulus; IBMX, 3-isobutyl-1-methylxanthine; IMCD, inner medullary collecting duct; mTAL, medullary thick ascending limb of Henle’s loop; OMCD, outer medullary collecting duct; PCT, proximal convoluted tubule; RBF, renal blood flow.
added after pH adjustment to 7.4 by NaOH. The collagenase solution (solution 2) was of the same composition as the microdissection solution, except it contained 0.1% BSA and 0.1% collagenase.

Isolation of nephron segments. Microdissection was done as previously described (7, 8) with a slight modification. Male Sprague-Dawley rats (120–170 g) were anesthetized with pentobarbital sodium (50 mg/kg i.p.). The left kidney was perfused in situ with 10 ml of solution 2 after clamping the thoracic aorta and right renal artery. The kidney was removed and 1-mm-thick slices were suspended in 15 ml of solution 2. They were incubated for 40 min at 37°C while 100% O₂ was bubbled through the solution. The slices were then washed with solution 1 and transferred to an ice-cold petri dish that was kept at 4°C by a cooling plate. Nephron segments were dissected under a dissecting microscope (Olympus, Tokyo, Japan). The isolated structures were glomerulus (Gl), proximal convoluted glomerulus and Henle’s loop (cTAL), medullary thick ascending limb of Henle’s loop (mTAL), cortical collecting duct (CCD), inner stripe of outer medullary collecting duct (OMCD), and middle third of the inner medullary collecting duct (IMCD). IMCD were dissected from carefully localized positions along the inner medullary axis (9). The total length of tubules was determined with an eyepiece micrometer. Microdissected tubules were transferred with 2 μl of solution 1 using a siliconized glass pipette into 1.5-ml plastic centrifuge tubes containing 18 μl of solution 1 with an appropriate concentration of IBMX.

Assay procedure of cAMP. Microdissected segments in 20 μl were preincubated with 10 μl of an appropriate concentration of endothelin and/or nicardipine, dioctanoyl glycerol, and H-7 for 10 min, indomethacin for 30 min at 37°C. After preincubation, 10 μl of an appropriate concentration of peptides in solution 1 with or without IBMX was added. The incubation was terminated after 3–10 min by the addition of 50 μl of ice-cold 10% TCA with complete mixing. Each tube was centrifuged for 5 min at 3,000 rpm. A 70-μl sample from the supernatant was stored at −40°C until the measurement of cAMP content. In each experiment, blank samples (containing medium and TCA without tubules) were also processed for cAMP standards. Frozen samples were thawed at room temperature and the TCA was removed by extraction four times with 1.0 ml of water-saturated ether. After evaporation of the ether, the remaining aqueous phase was dried using a vacuum drying oven (model DPA-30; Yamato Scientific Co., Ltd., Tokyo, Japan) and 100 μl of 50 mM sodium acetate buffer (pH 6.2) was added to each sample. Appropriate cAMP standards were added to blank samples. Samples of unknowns and cAMP standards were then assayed for cAMP content (radioimmunoassay kits; New England Nuclear, Boston, MA). The values of cAMP content were expressed per glomerulus or millimeter per 3 or 10 min. Although we used a very low concentration of Triton X-100 to dilute endothelin, Triton X-100 was further diluted according to the dilution of endothelin. At 0.000001% Triton X-100 does not affect the cAMP production stimulated by AVP. Each point is the mean value of two to three tubes.

Statistics. The results were given as means±SE. The differences were tested using analysis of variance or t test. P < 0.05 was considered significant.

Results

Effects of endothelin on AVP-dependent cAMP accumulation in rat distal nephron segments

Effects of IBMX in OMCDD. cAMP accumulation was about one-tenth in the absence of IBMX, compared with values obtained in the presence of 0.5 or 2.0 mM IBMX. AVP-dependent cAMP accumulation was similar in the presence of 0.5 and 2.0 mM IBMX. The effect of IBMX to inhibit phosphodiesterase in rat OMCDD seems maximal at a concentration of 0.5 mM (Fig. 1). Endothelin (10−8 M) inhibited AVP-dependent cAMP accumulation in both the presence and absence of IBMX: 55.4% in the absence of IBMX, 60.0% in the presence of 0.5 mM IBMX, and 51.1% in the presence of 2.0 mM IBMX. This result shows that the effect of endothelin on AVP-dependent cAMP accumulation is independent of phosphodiesterase. Therefore, the following experiments were done in the presence of 0.5 mM IBMX.

Time course in OMCDD. Endothelin (10−8 M) significantly reduced 10−9 M AVP-dependent cAMP production at all incubation times studied, from 1 to 10 min (Fig. 2). Effects of endothelin on cAMP accumulation induced by various concentrations of AVP in OMCDD. cAMP production by AVP reached a plateau at a concentration of 10−9 M (Fig. 3). The effect of endothelin became apparent at AVP concentrations from 1010 to 10−7 M. Endothelin (10−8 M) decreased cAMP production by 60%, from 36.8±5.1 to 14.8±2.6 fmol/mm per 3 min (n = 6, mean±SE, P < 0.01), at a concentration of 10−9 M AVP. At higher concentrations of AVP, the decrease in cAMP production by endothelin was ~ 30%. Therefore, we used 10−9 M AVP for further experiments.

Effect of various concentrations of endothelin at the fixed concentration of AVP (10−9 M) in CCD, OMCDD, and IMCDDD. Although the presence of endothelin binding was not shown in CCD by autoradiographic study (6), we investigated the effect of endothelin on AVP-dependent cAMP accumulation along the collecting duct, namely, CCD, OMCDD, and IMCDDD (Fig. 4). Endothelin dose dependently inhibited AVP-dependent cAMP accumulation in CCD from 65.5±4.4 to 43.5±2.8 (P < 0.001) and 31.3±2.9 fmol/mm per 3 min (n = 6, P < 0.001)
at concentrations of 10^{-10} and 10^{-8} M, respectively. Similarly, in OMCD AVP-dependent cAMP accumulation decreased from 36.8±5.1 to 21.2±3.2 and 14.8±2.6 fmol/mm per 3 min (n = 6, P < 0.01) at concentrations of 10^{-10} and 10^{-8} M endothelin. AVP (10^{-6} M)-stimulated cAMP production was about sixfold higher in IMCD compared with OMCD. cAMP production decreased by 23%, from 238.0±8.6 to 181.8±15.4 fmol/mm per 3 min (n = 6, mean±SE, P < 0.001) with 10^{-8} M endothelin. With 10^{-8} M of endothelin, cAMP production decreased by 56%.

Effect of Ca channel blocker nicardipine and indomethacin in OMCD. The effect of Ca channel blocker nicardipine was studied to investigate the mechanism of endothelin-induced inhibition of AVP-dependent cAMP accumulation (Fig. 5, left). Nicardipine enhanced AVP-dependent cAMP accumulation from 41.2±2.5 (n = 9) to 52.5±4.2 (n = 6, P < 0.02) and 56.6±3.4 fmol/mm per 3 min (n = 8, P < 0.01) at concentrations of 10^{-8} and 10^{-6} M, respectively. This effect is consistent with a previous report (14). Endothelin similarly inhibited AVP-dependent cAMP accumulation from the above values to 18.9±1.0 (n = 6) and 16.8±0.9 fmol/mm per 3 min (n = 10) in the presence of 10^{-8} and 10^{-6} M nicardipine, respectively. AVP did not induce cAMP accumulation with complete deletion of extracellular Ca by Ca-free solution containing 3 mM EGTA (data not shown).

Next, we studied the effect of indomethacin to see whether cyclooxygenase products, such as PGE\(_2\), mediate the inhibitory action of endothelin (Fig. 5, right). Endothelin had a similar inhibitory effect on AVP-dependent cAMP accumulation, from 40.0±3.6 (n = 6) to 19.1±1.6 (n = 6, P < 0.001) and 18.0±1.6 fmol/mm per 3 min (n = 6, P < 0.001) in the presence of 5 \times 10^{-7} and 5 \times 10^{-6} M indomethacin, respectively.

Effect of protein kinase C inhibitor H-7, 1-(5-isooquinolinesulfonyl)-2-methylpiperazine dihydrochloride, and dioctanoyl glycerol on OMCD. The effects of inhibition or activation of protein kinase C were investigated using H-7 and dioctanoyl glycerol. Protein kinase C inhibitor H-7 itself does not affect either basal or AVP-dependent cAMP accumulation (Fig. 6, left). AVP-dependent cAMP accumulation decreased from 40.7±2.1 (n = 6) to 19.6±2.4 fmol/mm per 3 min (P < 0.001) by endothelin (10^{-8} M) in the absence of H-7. However, AVP-dependent cAMP accumulation reversed almost to control values, to 34.4±4.4, 35.8±6.2, and 33.9±3.4 fmol/mm per 3 min in the presence of protein kinase C inhibitor H-7 at concentrations of 10^{-6}, 2.5 \times 10^{-5}, and 10^{-4} M, respectively. At
lower concentrations of H-7, effects of H-7 were partial (10^{-6} M) or lost (10^{-7} M).

The effect of protein kinase C activation by dioctanoyl glycerol is shown in Fig. 6 (right). Dioctanoyl glycerol mimicked the inhibitory effect of endothelin on AVP-dependent cAMP accumulation, from 38.8±2.1 \text{n} = 6 to 27.8±2.3 \text{n} < 0.01 and 21.5±1.5 fmol/mm per 3 min \text{n} < 0.001) at concentrations of 2.5 and 25 \mu g/ml, respectively.

Effect of endothelin in cTAL and mTAL. cAMP production is small in cTAL and mTAL compared with CCD, OMCD, or IMCD. Endothelin (10^{-8} M) had no significant effect on 10^{-9} M AVP-dependent cAMP accumulation in cTAL (from 5.7±0.6 \text{n} = 6 to 5.9±0.3 fmol/mm per 3 min) or mTAL (from 13.3±2.0 \text{n} = 6 to 9.7±2.0 fmol/mm per 3 min).

Effects of endothelin on PTH-, glucagon-, calcitonin-, and isoproterenol-dependent cAMP accumulation

Effect of endothelin on PTH-dependent cAMP accumulation in GI and PCT. Endothelin (10^{-8} M) had no effect on PTH-dependent cAMP accumulation in GI, where binding of endothelin was shown in an autoradiographic study (6) (from 18.7±1.8 to 19.6±1.3 fmol/GI per 3 min \text{n} = 6 and from 10.3±1.1 to 10.7±1.1 fmol/GI per 3 min at 10^{-7} and 10^{-8} M of PTH), respectively. The effect of endothelin was also studied in PCT, where the main target segment of PTH is located. However, endothelin (10^{-8} M) had no effect on PTH-dependent cAMP accumulation in PCT (from 26.4±2.2 to 29.0±2.1 fmol/mm per 3 min \text{n} = 6, and from 9.0±0.9 to 9.2±1.2 fmol/mm per 3 min \text{n} = 6) at 10^{-6} and 10^{-8} M of PTH, respectively.

Effects of endothelin on glucagon-, calcitonin-, and isoproterenol-dependent cAMP accumulation in OMCD. Endothelin (10^{-8} M) had no effect on glucagon-dependent cAMP accumulation in OMCD (from 7.4±1.2 to 7.3±1.0 fmol/mm per 10 min \text{n} = 6, and from 2.8±0.4 to 2.9±0.2 fmol/mm per 10 min \text{n} = 6) at 10^{-4} and 10^{-8} M glucagon, respectively. Calcitonin-dependent cAMP accumulation was not affected either (from 4.6±0.9 to 4.2±0.4 fmol/mm per 10 min \text{n} = 6, and from 1.8±0.2 to 1.9±0.1 fmol/mm per 10 min \text{n} = 6) at 10^{-7} and 10^{-8} M calcitonin.

Isoproterenol-dependent cAMP accumulation was not affected (from 1.8±0.2 to 1.7±0.2 fmol/mm per 10 min \text{n} = 6, and from 1.4±0.2 to 1.2±0.2 fmol/mm per 10 min \text{n} = 6) at 10^{-6} and 10^{-8} M isoproterenol, respectively.

Discussion

We investigated the interaction between endothelin and peptide-dependent cAMP accumulation in rat nephron segments. Our results indicated that endothelin dose dependently inhibited AVP-dependent cAMP accumulation in CCD, OMCD, and IMCD. Endothelin did not have an inhibitory effect on AVP-dependent cAMP accumulation in cTAL and mTAL. The inhibitory effect of endothelin in peptide-dependent cAMP accumulation is considered to be specific to AVP and collecting ducts because endothelin had no effect on PTH-dependent cAMP accumulation in GI and PCT, or on glucagon-, calcitonin-, and isoproterenol-dependent cAMP accumulation in OMCD.

Several mechanisms can be considered for the inhibition of AVP-dependent cAMP accumulation by endothelin: for example, competitive inhibition of receptor, the stimulation of phosphodiesterase activity, and the inhibition of adenylate cyclase. There are no reports so far that endothelin competitively inhibits the receptor of AVP. Therefore, this mechanism is less likely. It is not clear why endothelin inhibits AVP-dependent cAMP production less in supermaximal AVP stimulation than in maximal stimulation. A more prolonged preincubation time or a higher concentration of endothelin might be necessary to inhibit the effect of AVP in case of a supermaximal concentration of AVP. The possibility that endothelin stimulates phosphodiesterase activity is small, because the inhibitory effect of endothelin on AVP-stimulated cAMP production is independent of the presence or absence of phosphodiesterase inhibitor (IBMX) (Fig. 1). It is more likely that endothelin has an inhibitory effect on the adenylyl cyclase activity. α₂-Adrenergic stimulators are also known to inhibit cAMP production in collecting ducts, probably through the activation of inhibitory G protein (8, 10, 11).

The mechanism by which endothelin inhibits adenylyl cyclase activity is not known. As the mechanism of endothelin action, the increments of intracellular Ca are required in smooth muscle cells or cultured mesangial cells (1, 12). The influx of Ca into cytoplasm is known to inhibit the cAMP production, possibly through the inhibition of a catalytic subunit of adenylyl cyclase (13, 14). In our experiment, Ca channel blocker nicardipine had no effect on endothelin-induced inhibition of AVP-dependent cAMP accumulation. Therefore, endothelin inhibited AVP-dependent cAMP accumulation through other mechanisms than the influx of extracellular Ca. A report suggests that endothelin inhibits Na-K-ATPase in IMCD cells of rabbits (15), possibly mediated by cyclooxygenase products such as PGE₂, although indomethacin had no effect on endothelin action in smooth muscle cells (1). From our results, endothelin had a similar inhibitory effect even in the presence of indomethacin, suggesting that cyclooxygenase products such as PGE₂ do not mediate the inhibitory action of endothelin. One of the reasons for the difference may be that there are species differences in the effect of PGE₂ on AVP-induced cAMP accumulation between rat and rabbit CCD (16). In the rat CCD, PGE₂ (3 \times 10^{-7} M) had no effect on AVP-dependent cAMP accumulation.

Endothelin was shown to stimulate a sustained increase in 1,2-diacylglycerol and protein kinase C activation in bovine aortic smooth muscle cells (17). The endothelin-induced constriction was reversed by the protein kinase C inhibitor H-7 (18). In our results, the inhibitory effect of endothelin was abolished by H-7, and the effect of endothelin was mimicked by protein kinase C activator dioctanoyl glycerol, suggesting that protein kinase C activation is involved in the inhibitory effect of endothelin on AVP-dependent cAMP accumulation in the collecting duct. Endothelin is not a toxic substance because the effect of AVP was reversed by H-7 in the presence of endothelin. The role of protein kinase C in inhibiting AVP-dependent cAMP accumulation was also reported in cultured collecting tubular cells (19).

Endothelin increased the urine flow rate despite a decrease in GFR and renal blood flow (RBF) in the rat (4). Another report showed that endothelin increased free water clearance and decreased urinary sodium excretion, urine osmolality, and osmolar clearance in the presence of an unchanged or increased concentration of plasma AVP (5). A decrease in GFR and RBF may be enough to explain the decrease in urinary sodium excretion, but it cannot explain the increase in free water excretion. In collecting ducts, AVP stimulates water permeability by stimulating cAMP generation. Our data demon-
strating that endothelin inhibits AVP-dependent cAMP accumulation in CCD, OMCD, and IMCD, but not in cTAL and mTAL, although we cannot rule out the possibility that endothelin affects cTAL and mTAL through other pathways.

Endothelin stimulates atrial natriuretic peptide (ANP) and cAMP accumulation in CCD, OMCD, and IMCD, but not in cTAL and mTAL. This effect was possibly due to the inhibition of adenylate cyclase activity by protein kinase C activation, and was independent of phosphodiesterase, Ca channel, or cyclooxygenase products. On the other hand, endothelin had no effect on PTH-dependent cAMP accumulation in GI and PCT, and glucagon-, calcitonin-, and isoproterenol-dependent cAMP accumulation in OMCD. Our data suggest that endothelin works to increase water excretion by inhibiting the AVP effect on collecting ducts. This explains, at least partly, the increase in urinary volume in the presence of a decrease in GFR and renal plasma flow caused by endothelin.

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