UNIQUE CONTRACTILE ACTION OF ENDOTHELINS ON PORCINE ISOLATED URETER AND CHARACTERIZATION OF THE ENDOTHELIN-BINDING SITES

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ABSTRACT

Endothelin produced rhythmic contractions in porcine isolated ureter, which was sensitive to nitrendipine and EDTA. Dose response studies indicated that the rhythmic contractile response of the ureter became apparent at 1 nM and its frequency increased markedly with increasing concentrations of endothelin-1. Endothelin-2 and endothelin-3 were also equally effective. To characterize the endothelin-binding sites on the ureter, we carried out affinity labeling and competitive binding assays. Affinity labeling with $^{125}$I-endothelin-1 yielded one major (60 K) and two minor (36 K and 31 K) bands; when $^{125}$I-endothelin-3 was used, only 60 K and 31 K bands were labeled. Competitive binding assay using radiolabeled endothelin-1 and endothelin-3 revealed that endothelin-1 was much more effective than endothelin-3 in displacing $^{125}$I-endothelin-1; similarly, endothelin-3 was more potent than endothelin-1 in displacing $^{125}$I-endothelin-3, indicating that there are at least two distinct endothelin receptors in the ureter: one which is specific for endothelin-1 and the other specific for endothelin-3.

Endothelin, a recently discovered vasoconstrictor peptide, has at least three distinct isoforms: endothelin-1, endothelin-2 and endothelin-3 (6, 13). Physiological roles of each isoform, tissue distribution of specific receptors and the mechanism of action have been investigated extensively. Using rat uterus, we demonstrated that endothelin could contract not only vascular smooth muscles but also nonvascular smooth muscle cells (5, 8) and endothelin activated phospholipase C and phospholipase A$_2$ signal transduction pathways via specific receptors (7), suggesting that such nonvascular tissues may also serve as potentially useful materials for elucidating the mode of action of endothelin. Tissue selectivity of endothelin was also examined by Borges et al. (1), Bousso-Mittler et al. (2), Geppetti et al. (3), Maggi et al. (10) and Secrest and Cohen (12) using vascular and nonvascular smooth muscle preparations such as isolated uterus (1, 2), trachea (1, 12), vas deferens (1), iris (3) and urinary bladder (10, 12). While extending the tissue selectivity analysis to other nonvascular smooth muscles, we found that the ureter is very sensitive to all three members of the endothelin family and contracts rhythmically; this mode of endothelin-induced contractions is very unique since most smooth muscles have been shown to respond to endothelins by either slowly developing sustained contractions as typically seen in vascular smooth muscles (13) or a combination of rhythmic and developing contractions as in uterus (1, 5, 8). Biochemical characterization of the endothelin-binding sites revealed that ureter smooth muscles contain two distinct receptor subtypes that translate the binding of endothelins into coordinated mechanical activity.

MATERIALS AND METHODS

Materials

Endothelin-1, endothelin-2 and endothelin-3 were purchased from Peptide Institute, Osaka. $^{125}$I-Tyr$^{13}$-
Endothelin-1 (~74 TBq/mmol) and $^{125}$I-Tyr$^6$-endothelin-3 (~74 TBq/mmol) were purchased from Amersham. Nitrendipine was obtained from Yoshitomi Pharmaceutical Industries, and protected from light during the experiments. Disuccinimidyl suberate (DSS) was from Pierce.

**Measurement of Contractile Activity**

Fresh porcine ureter and urinary bladder were obtained from a local slaughterhouse. Helically cut strips (15 mm long) of ureter and strips (20 mm long) from upper portions of urinary bladder freed from mucosa were mounted in a 10-ml organ bath containing Krebs-Henseleit solution maintained at 37°C and saturated with 95% O$_2$ and 5% CO$_2$ under a resting tension of 1 g, and were challenged two or more times with 30 mM KCl until reproducible response was obtained. The contractions were recorded using an isometric transducer (KN-259) and a recorder (KN-260) from Natsume Seisakusho, Tokyo.

**Binding Assay**

Endothelin-binding activity of porcine ureteral membranes was measured using $^{125}$I-endothelin-1 and $^{125}$I-endothelin-3 as described previously (5). Porcine ureter was homogenized with a Waring blender in 20 mM phosphate buffer, pH 7.5, containing 130 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 10 μg/ml pepstatin, and 10 μg/ml leupeptin, washed twice by centrifugation at 10,000 g for 15 min at 4°C, and resuspended in the same buffer, and used as membrane suspension. For the competitive binding assay, 50 μl of $^{125}$I-endothelin-1 (920 Bq) or $^{125}$I-endothelin-3 (920 Bq) and various concentrations of unlabeled endothelins were added to 100 μl of the above membrane suspension (50 μg protein). The mixture was incubated for 30 min at 30°C and then free radioactive ligand was removed by centrifugation. Radioactivity was measured with an Aloka gamma counter (ARC-300). Nonspecific binding was determined in parallel experiments in the presence of an excess (1 μM) of
unlabeled endothelins and subtracted from total binding to obtain the specific binding. Nonspecific binding of $^{125}$I-endothelin-1 was less than 25%; in the case of $^{125}$I-endothelin-3, it was relatively high (50–65%).

**Affinity Labeling**

Membrane suspensions (25 µg protein/50 µl) were incubated with 20 µl of $^{125}$I-endothelin-1 (9.2 kBq) or $^{125}$I-endothelin-3 (9.2 kBq) for 30 min at 30°C in the presence or absence of corresponding unlabeled endothelins (1 µM), and then cross-linking reagent DSS (50 mM in dimethyl sulfoxide) was added to a final concentration of 1 mM. After a 20 min incubation at 25°C, 20 µl of 2 M ammonium acetate were added to the mixtures to stop the cross-linking reaction. The mixtures were subjected to SDS-PAGE. Radioligand/receptor complexes cross-linked with DSS were electrophoresed on 10% polyacrylamide slab gels under reducing conditions according to Laemmli (9). After electrophoresis, proteins in the gels were fixed and the gels were dried in a gel drier and exposed to Kodak X-Omat AR film at −70°C for 5 days.

**RESULTS**

**Contractile Action of Endothelins on Porcine Isolated Ureter**

Fig. 1a shows the responses of porcine isolated ureter to endothelin-1 and endothelin-3. Both endothelin-1 and endothelin-3 caused concentration-dependent rhythmic contractions in the presence of extracellular calcium. The effect appeared at a concentration as low as 1 nM, and with increasing concentrations of endothelins the frequency of the rhythmic contractions increased. The rhythmic contractions were completely abolished by a voltage-dependent calcium channel blocker (nitrendipine) and a calcium chelating reagent (EDTA). As expected from the close structural similarity between endothelin-1 and endothelin-2 (they differ only in 2 amino acid residues out of 21; endothelin-3 has 6 different amino acids compared to endothelin-1), endothelin-2 showed nearly equivalent activity (data not shown).

To examine tissue specificity of the response, the effect of endothelins on urinary bladder was also determined (Fig. 1b); at 10 nM, endothelin-1 evoked a sustained contraction in smooth muscles of porcine isolated urinary bladder, which was only partially inhibited by nitrendipine and EDTA. Endothelin-3 was less effective, showing its contractile activity at concentrations higher than 100 nM.

**Biochemical Characterization of Ureter Endothelin Receptors**

Fig. 2 shows the autoradiogram of porcine ureteral
Fig. 3 Binding of $^{125}$I-endothelin-1 (A) and $^{125}$I-endothelin-3 (B) to porcine ureteral membranes in the presence of unlabeled endothelins. The membranes were incubated with 12.5 fmol (920 Bq) of $^{125}$I-endothelin-1 (A) or 12.5 fmol (920 Bq) of $^{125}$I-endothelin-3 (B) in the presence of various concentrations of unlabeled endothelin-1 (●) and endothelin-3 (○). Each point represents the mean of duplicate measurements.

receptors covalently tagged with $^{125}$I-endothelin-1 or $^{125}$I-endothelin-3. Specific receptor bands were identified, among many non-specifically labeled bands that disappeared in the presence of an excess of unlabeled endothelins (1 μM). Affinity labeling of ureteral membranes with $^{125}$I-endothelin-1 yielded one major band of 60 K and two minor bands of 31 K and 36 K (lanes a and b). On the other hand, the labeling profiles obtained with $^{125}$I-endothelin-3 demonstrated only 31 K and 60 K bands (lanes c and d). We assumed the bands marked by asterisks (●) as nonspecific although their intensities were significantly reduced by the addition of unlabeled endothelins, because i) these bands did not completely abolish in the presence of 1 μM unlabeled endothelins, and ii) their positions coincided with the major protein bands on the Coomassie blue-stained SDS-PAGE gels; usually, such abundant proteins are labeled nonspecifically.

We next examined the competition of $^{125}$I-endothelin-1 and $^{125}$I-endothelin-3 binding to porcine ureteral membranes with unlabeled endothelins. $^{125}$I-Endothelin-1 binding was competitively inhibited by increasing concentrations of unlabeled peptides in the following order: endothelin-1 > endothelin-3 (Fig. 3A). In contrast, the order of potency in displacing $^{125}$I-endothelin-3 binding was endothelin-3 > endothelin-1 (Fig. 3B). These results and the biphasic displacement of $^{125}$I-endothelin-1 with endothelin-3 (Fig. 3A, open circle) clearly indicate that there are at least two groups of binding sites which are distinguishable in their affinities for endothelin-3. For endothelin-1, however, the two groups show almost identical affinities as seen by comparing curves ET-1 (closed circle) in Figs. 3A and 3B; the $K_D$ values of endothelin-1 for inhibiting $^{125}$I-endothelin-1 (Fig. 3A, ●) and $^{125}$I-endothelin-3 (Fig. 3B, ○) were both roughly 0.1 nM. Scatchard analysis of the Fig. 3 data revealed that i) the endothelin-3-specific site had a $K_D$ of 7 pM for endothelin-3 (0.1 nM for endothelin-1) and $B_{max}$ of 25 fmol/mg of protein and ii) the other site had a $K_D$ of 0.1 nM for endothelin-1 (50 nM for endothelin-3) and $B_{max}$ of 65 fmol/mg of protein.

**DISCUSSION**

Activation of smooth muscle cells by endothelin is chiefly caused by an increase in intracellular calcium level (4, 11). Our results concerning the effects of nitrendipine, a voltage-dependent calcium channel antagonist, and EDTA indicate that the endothelin-induced rhythmic contractions of ureter (Fig. 1a) are produced by the influx of extracellular calcium through the voltage-dependent calcium channels and the monophasic developing contractions of urinary bladder (Fig. 1b) are mainly produced by the release of calcium from the intracellular calcium store. Moreover, we indicated that the signal transduction pathways of endothelins in ureter smooth muscle cells were initiated by the specific receptors (Fig. 2). Competitive binding assays using endothelin-1 and endothelin-3 revealed differences in displacing radioligands; endothelin-3 effectively competed with $^{125}$I-endothelin-3 but less effective in inhibiting $^{125}$I-endothelin-1 binding (Fig. 3). These results suggest that there are at least two receptor subtypes in the ureter: one which is specific for endothelin-1 and one which is specific for endothelin-3.

Phenomenally, endothelin-responsive smooth muscles seem to be classified into three types: 1) a group showing only monophasic sustained contractions in response to a wide range of endothelin concentrations; 2) a group in which both rhythmic and slowly developing contractions occur; and 3) as newly identified here using porcine ureter, smooth muscles that exhibit rapid rhythmic contractions alone. Physiological relevance of these different types of responses to endothelins is not clear at present; however, in the case of the ureters, their
ENDOTHELIN-INDUCED URETERAL CONTRACTION

Rhythmic contractions seem to be quite consistent with their role to transport urine down their length by peristaltic movements, thereby suggesting the contractile effects of endothelins on the ureter may be of a physiological significance.

Endothelin-induced contractions of ureter are unique in their 1) rhythmic nature devoid of monophasic developing contractions, and 2) equal sensitivity to endothelin-1, endothelin-2 and endothelin-3. Further analyses using cultured ureteral smooth muscle cells would be useful to understand the molecular basis of the unique action of endothelins on the ureter.

This work was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, the Mitsubishi Foundation, Chichibu Cement and the Japan Shipbuilding Industry Foundation.

Received 17 October 1990; accepted 3 December 1990

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