PROPERTIES OF RAT UTERUS ENDOTHELIN RECEPTOR SITES

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ABSTRACT

Endothelin-binding sites in rat uterus have been characterized in terms of ligand specificity and molecular weight. Contractile responses of uterus horns to endothelin analogues were first examined; endothelin-1 and endothelin-2 caused rhythmic contractions at 1 nM and developing monophasic contractions at 10 nM; however, in the case of endothelin-3, much higher concentrations were required to induce the rhythmic (50 nM) and developing (500 nM) contractions. These responses are unique since in the vascular system all the three members of the endothelin have been demonstrated to be active at subnanomolar concentrations. Consistent with these results of physiological response analysis, binding assays using radioligands revealed that rat uterus bound ^{125}I -endothelin-1 with a high affinity but it showed only a weak affinity for ^{125}I -endothelin-3. The rank order of binding potency determined by competitive binding assay was: endothelin-1 $\geq$ endothelin-2 $\gg$ endothelin-3. Affinity labeling of the binding sites on the uterus membranes using ^{125}I -endothelin and the cross-linking reagent 1,5-difluoro-2,4-dinitrobenzene showed one major band with an M_r of 48,500. These results indicate that the rat uterus endothelin receptor site is a 46 K membrane protein and specific for endothelin-1 and endothelin-2.

The endothelins are members of a newly discovered family of vasoactive peptides that contain two conserved disulfide bridges in a 21-residue structure. Endothelin-1, endothelin-2, and endothelin-3, which are mammalian peptides, bear significant homologies to snake venom sarafotoxin S6B (5, 8, 13). These peptides modulate cardiovascular tone by a direct action on smooth muscle cells that requires influx of extracellular calcium (17). Endothelin also exerts profound effects on nonvascular smooth muscle cells; we have recently demonstrated that endothelin-1 causes rhythmic contractions of rat uterus at subnanomolar concentrations (6).

Recent identification of new members of the endothelin family (i.e., endothelin-2 and endothelin-3) by Inoue *et al.* (4) and classifica-

tion of the endothelin-binding sites on chick cardiac muscles into two subtypes by Watanabe *et al.* (16) prompted us to characterize the rat uterus endothelin-binding sites using the new members of endothelin, endothelin-2 and endothelin-3. The results indicated that in contrast to the cardiac muscles which contain a mixed population of endothelin-1/endothelin-2-specific and endothelin-3-specific binding sites, the uterus has only endothelin-1/endothelin-2-specific binding sites, whose M_r was determined to be 46,000 by affinity labeling.

MATERIALS AND METHODS

Materials

^{125}I -Tyr 13 Endothelin-1 (~ 74 TBq/mmol) and ^{125}I -Tyr 6 endothelin-3 (~ 74 TBq/mmol) were

purchased from Amersham. Endothelin-1, endothelin-2, and endothelin-3 were from Peptide Institute (Osaka). 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) and disuccinimidyl suberate (DSS) were from Pierce. Other chemicals were of analytical grade.

Measurement of Uterus Contraction

The contractile activity of rat uterine horns was measured according to the method described previously (6). The uteri were obtained from Wister rats (150 g) injected with 0.2 mg of β -estradiol in the peritoneum 24 h before experiments. Each uterus horn (2–3 cm) was mounted in a 10-ml organ bath containing Munsick buffer (113 mM NaCl, 6 mM KCl, 0.5 mM CaCl_2 , 1 mM NaH_2PO_4 , 30 mM NaHCO_3 , and 1.6 mM glucose) maintained at 37°C and saturated with 95% O_2 and 5% CO_2 under a resting tension of 1 g. 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 uterus homogenate was measured using ^{125}I -endothelin-1 and ^{125}I -endothelin-3 as described previously (6). Two rat uteri were homogenized in 50 ml of 20 mM phosphate buffer, pH 7.5, containing 130 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ pepstatin and 10 $\mu\text{g}/\text{ml}$ leupeptin, washed twice, and resuspended in 40 ml of the same buffer, and used as plasma membrane preparation. Various concentrations of ^{125}I -endothelin-1 were added to 500 μl of the above membrane suspension in the presence and absence of 1 μM unlabeled endothelin-1. 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). For the competitive binding assays, various concentrations of unlabeled endothelin analogues and 920 Bq of ^{125}I -endothelin-1 or ^{125}I -endothelin-3 were added to the incubation mixture. Using ^{125}I -endothelin-3, another series of the competitive binding assay was carried out in the presence of low concentrations (10 nM) of endothelin-1 in order to determine whether

^{125}I -endothelin-3 binds to its specific sites or to endothelin-1/endothelin-2 receptor sites.

Affinity Labeling

Detergent extracts of rats uterus membranes were prepared as follows. 3[(3-Cholamidopropyl)dimethylamino] - 1 - propanesulfonate (CHAPS) was added to the membrane suspensions (10 mg protein/ml) until the concentration reached 0.25% (w/v). The mixture was stirred for 2 h at 4°C and then was centrifuged at 100,000 g for 1 h at 4°C. The extracts (50 μl each) were incubated with 50 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, and then cross-linking reagents DFDNB or DSS (50 mM in dimethylsulfoxide) was added to a final concentration of 1 mM. The mixture was incubated for 20 min at 25°C and 20 μl of 2 M ammonium acetate was added to stop the cross-linking reaction. The mixture was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

Radioligand-receptor complexes cross-linked with DFDNB and DSS were boiled in 10 mM Tris-HCl, pH 6.8, containing 1% SDS, 20% glycerol, 1% 2-mercaptoethanol, and 0.1% bromophenol blue and electrophoresed on 10% polyacrylamide slab gels under reducing conditions according to Laemmli (7). 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 10 days.

RESULTS AND DISCUSSION

Uterine Smooth Muscle Responds to Endothelin-1 and Endothelin-2 But Not to Physiological Concentrations of Endothelin-3

We previously demonstrated that endothelin-1 causes two types of contractions of rat uterus, namely 1) phasic contractions sensitive to voltage-dependent calcium channel blockers, and 2) monophasic developing contractions insensitive to the calcium channel blockers (dihydropyridine antagonists) (6). These

Fig. 1 Typical contractile responses of rat uterus to endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3). Uterus, of which contractile ability was confirmed by oxytocin, was used in this experiment. Endothelin (ET-1, ET-2, or ET-3) was added to the bath at the point indicated by arrows $\uparrow$. $\blacktriangle$: uterus was rinsed with Munsick buffer.

responses are shown, as controls, in Fig. 1a. Responses of rat uterine horns to endothelin-2 and endothelin-3 are depicted in Fig. 1, b and c, respectively. Endothelin-2 behaved similarly as endothelin-1, inducing the two types of contractions: rhythmic contractions at 1 nM and developing contractions at 10 nM. Endothelin-3, however, had no effect even at 10 nM; its effects became apparent at more than 50 times higher concentrations than those of endothelin-1 and endothelin-2. Taking it into account that endothelin-3 has been shown to exert profound physiological actions on coronary artery (4) and on other vasculatures (14), these results suggest that 1) there exist at least two distinct endothelin receptor systems (endothelin-1/endothelin-2-specific and endothelin-3-specific systems) in the rat, 2) the uterus has only the former, and 3) although the uterus receptor is specific for endothelin-1 and endothelin-2, it also shows a weak affinity for endothelin-3 and can therefore be activated with high concentrations of

Fig. 2 Ligand specificity of endothelin (ET)-binding sites determined by competitive binding assay. Rat uterus membranes (200 μ g/500 μ l) were incubated at 30°C for 30 min with 125 I-endothelin-1 (920 Bq/tube) or 125 I-endothelin-3 (920 Bq/tube) in the presence of various concentrations of unlabeled endothelins. Curves a, b, and c represent displacement of 125 I-endothelin-1 with unlabeled endothelin-1, endothelin-2, and endothelin-3, respectively. Curves d and e displacement of 125 I-endothelin-3 with varying concentrations of unlabeled endothelin-3 in the absence and the presence of a low concentration of endothelin-1 (10 nM), respectively. Values are the means of four different determinations.

endothelin-3. To confirm this speculation, we carried out binding assays using radioiodinated endothelin-1 and endothelin-3.

Uterine Binding Sites for Endothelin Are Specific for Endothelin-1 and Endothelin-2

Fig. 2 shows displacement of 125 I-endothelin-1 from its binding sites on rat uterus membranes with unlabeled endothelin-1 (curve a), endothelin-2 (curve b), and endothelin-3 (curve c). The binding of 125 I-endothelin-1 was competitively inhibited by endothelin-1 ($K_i = 0.3 \pm 0.02$ nM) and endothelin-2 ($K_i = 1.0 \pm 0.1$ nM); however, only weak competition was observed with endothelin-3 ($K_i = 0.4 \pm 0.08$ μ M). These results of competitive binding assay are in good agreement with the above physiological response analysis. Curves d and e show the binding of 125 I-endothelin-3 to rat uterus membranes in the presence of various concentrations of unlabeled endothelin-3. Very little specific binding of 125 I-endothelin-3

Fig. 3 Binding of ^{125}I -endothelin-1 to rat uterus membranes. Rat uterus membranes ($200\ \mu\text{g}/500\ \mu\text{l}$) were incubated at 30°C for 30 min with various concentrations of radioiodinated endothelin-1. Binding of ^{125}I -endothelin-1 to rat uterus membranes in the presence ($\circ$, NS) and the absence ($\bullet$, T) of $1\ \mu\text{M}$ unlabeled endothelin-1. Nonspecific binding was less than 20% of total binding. Specific binding was defined as total binding ($\bullet$, T) minus nonspecific binding ($\circ$, NS). Scatchard plot of specific binding of ^{125}I -endothelin-1 is shown in the inset.

was observed, which was diminished in the presence of low concentration of endothelin-1 ($10\ \text{nM}$) (curve e) or by the addition of an excess of unlabeled endothelin-3 (curve d). To eliminate the possibility that the poor binding of ^{125}I -endothelin-3 to uterus membranes is due to the modification of the tyrosine of endothelin-3 with bulky ^{125}I rather than due to lack of the specific binding sites, we examined binding of ^{125}I -endothelin-3 to brain and lung membranes as control experiments. ^{125}I -Endothelin-3 bound to these membranes equally well as ^{125}I -endothelin-1, indicating that radioiodination does not impair the binding activity of endothelin-3.

Binding data obtained with ^{125}I -endothelin-1 are presented in Fig. 3. Relatively large amounts ($B_{\text{max}} = 150 \pm 50\ [\text{SE}]\ \text{fmol/mg}$ of protein, $n=3$) of ^{125}I -endothelin-1 bound to uterus membranes with a high affinity ($K_D = 0.12 \pm 0.03\ \text{nM}$); the affinity and the number of the binding sites for endothelin-1 were calculated from the Scatchard plot shown in Fig. 3 inset.

These results indicate that rat uterus pos-

sesses an endothelin-responsive system specific for endothelin-1 and endothelin-2 and therefore should serve as an ideal material for studying the properties of endothelin-1/endothelin-2-specific binding sites since many of other tissues including brain, lung, and heart (4, 14, 15) have a mixed population of endothelin-1/endothelin-2-specific and endothelin-3-specific binding sites.

Affinity Labeling Revealed an $M_r=46,000$ Protein as a Target of Endothelin-1 and Endothelin-2

Endothelin-binding sites were solubilized from rat uterus membranes with 0.25% CHAPS, complexed with radioiodinated endothelin-1, endothelin-2, or endothelin-3, and cross-linked with bifunctional reagents DFDNB and DSS which have 0.3 and 1.1 nm span, respectively. When analyzed by SDS-PAGE, the receptor sites affinity-labeled with ^{125}I -endothelin-1 and DFDNB migrated as a 48.5 K band (Fig. 4, lane a). The specificity of the labeling was supported by the absence of 48.5 K band in the control sample prepared in the presence of excess unlabeled endothelin-1. As expected, a similar result was obtained with ^{125}I -endothelin-2 (data not shown). Also consistent with the results of binding assays, cross-linking experiments with ^{125}I -endothelin-3 yielded no specifically labeled band except a very faint band of 48.5 K which may represent a product of the weak interaction between ^{125}I -endothelin-3 and the endothelin-1/endothelin-2 receptor sites as demonstrated in the preceding section.

When DSS was used instead of DFDNB as a cross-linking reagent, the affinity-labeled protein migrated slightly faster than the DFDNB-treated sample, yielding a band of 43 K. This slight difference in the electrophoretic mobility may be due to intramolecular cross-linking of the target protein with DSS as explained below; bifunctional reagents can form covalent linkages not only between a ligand and a receptor protein but also between two amino groups within a protein molecule if the amino groups are properly spaced so as to match the span of the cross-linkers; and once the intramolecular cross-linking occurs, the cross-linked protein would not unfold completely even in the presence of SDS and hence would

Fig. 4 Autoradiograms showing affinity-labeled endothelin-1-binding sites in rat uterus. Detergent extracts of uterus membranes were incubated with ^{125}I -endothelin-1 (lanes a, b, e, and f) or ^{125}I -endothelin-3 (lanes c and d) in the presence and the absence of an excess of unlabeled endothelins, and radioligand-receptor complexes were cross-linked with 1 mM DFDNB (lanes a-d) and DSS (lanes e and f). Samples were electrophoresed under reducing conditions on 10% SDS-polyacrylamide gels and radioactive bands were visualized by fluorography. Arrows indicate the positions of 48.5 K (DFDNB) and 43 K (DSS) bands specifically radiolabeled with ^{125}I -endothelin-1 or ^{125}I -endothelin-3. The bands that were not abolished with an excess of unlabeled endothelin were considered nonspecific. Molecular weight markers used were: β -galactosidase (116 K), phospholipase b (97 K), bovine serum albumin (68 K), egg albumin (45 K), and carbonic anhydrase (29 K). Similar results were obtained when ^{125}I -endothelin-2 was used instead of ^{125}I -endothelin-1 (data not shown).

run faster than the uncross-linked species on SDS-PAGE. Therefore, we estimated the M_r of endothelin-1/endothelin-2-binding sites to be 46,000 by subtracting the M_r of endothelin from the value 48,500 obtained with DFDNB.

Affinity labeling using plasma membranes of rat uterus, prepared as described in Materials and Methods, also resulted in identification of the $M_r=46,000$ species as the endothelin-1/endothelin-2-binding site (data not shown).

Two mechanisms have been proposed for the action of endothelin: one involves a direct action of endothelin on voltage-dependent calcium channels (3, 17) and the other involves receptor-mediated stimulation of phosphoinositide system (5, 11, 14). The affinity labeling results reported here indicate that if direct opening by endothelin of the voltage-dependent calcium channel occurs as proposed in the first mechanism, it should be through the binding of endothelin to the β -

subunit (50 K) of the channel since the other components (α , γ , and δ subunits) are much larger or smaller (2) than the affinity-labeled 46 K species. However, this interaction seems to be difficult to occur because the β -subunit has been proposed to be located on the cytosolic surface of the plasma membranes (12). Recently, Miyazaki *et al.* (10) and Martin *et al.* (9) have demonstrated, in chick cardiac muscle and rat glomeruli, the presence of specific endothelin receptor that is clearly distinguishable from the voltage-dependent calcium channel. This strongly supports the receptor mechanism. The 46 K membrane protein identified in the present study may also represent specific uterine receptor for endothelin.

The biochemical basis for the biphasic response shown in Fig. 1 should be addressed in future studies by, for example, clarifying whether the receptor demonstrated in the present study is responsible for mediating the

rhythmic contractions or the continuous contractions.

After completion of this work, Mittler *et al.* (1) have reported a similar selective binding of endothelin-1 to the rat uterus.

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