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ENDOTHELIN INDUCES TWO TYPES OF CONTRACTIONS OF RAT UTERUS: PHASIC CONTRACTIONS BY WAY OF VOLTAGE-DEPENDENT CALCIUM CHANNELS AND DEVELOPING CONTRACTIONS THROUGH A SECOND TYPE OF CALCIUM CHANNELS

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Effects of endothelin on nonvascular smooth muscle have been examined using rat uterine horns and two modes of endothelin action have been revealed. Endothelin (0.3 nM) caused rhythmic contractions of isolated uterus in the presence of extracellular calcium. The rhythmic contractions were completely inhibited by calcium channel antagonists. These characteristics of endothelin-induced contractions were very similar to those induced by oxytocin. Binding assays using ^{125}I-endothelin showed that endothelin and the calcium channel blockers did not compete for the binding sites. However, endothelin was unique in that it caused, in addition to rhythmic contractions, a slowly developing monophasic contraction that was insensitive to calcium channel blockers. This developing contraction became dominant at higher concentrations of endothelin and was also calcium dependent.

Endothelin is a potent vasoconstrictor isolated from culture media of porcine aortic endothelial cells (1) and considered to be an important modulator of vascular tone. It is a hydrophobic peptide consisting of 21 amino acids and has an unique activity to cause a slow and sustained contraction of vascular smooth muscle. In relation to its structural homologies to ion channel toxins of scorpion (2), bee (3), and snake (4), the mechanism of action of endothelin has attracted the interest of many researchers: Yanagisawa *et al.* (1) suggested the possibility that endothelin is a direct modulator of voltage-dependent calcium channels; on the other hand, Auguet *et al.* (5) reported that endothelin contraction of rat aorta was not inhibited by calcium

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antagonists; Hirata et al. (6) demonstrated that calcium channel blockers did not affect the binding of endothelin to cultured vascular smooth muscle cells. While examining the effects of endothelin on nonvascular smooth muscle, we found that rat uterus is a useful material to investigate the contractile properties of endothelin. The uterus responded to endothelin in two ways, namely by showing rhythmic contractions and a slowly developing monophasic contractions. These two responses occurred simultaneously, but could clearly be distinguished by their sensitivity to voltage-dependent calcium channel antagonists, indicating that endothelin exerts its effects through multiple pathways.

MATERIALS AND METHODS

Materials

Endothelin was purchased from Peptide Institute, Oosaka, Japan, and was dissolved in a 0.015% Triton X-305 solution, stored at 4°C until assay. Nifedipine and nitrendipine were protected from light during the experiments. ^{125}I-endothelin (2000 Ci/m mol) was obtained from Amersham UK.

Measurement of Uterine Contractions

The contractile activity was measured by using rat uterine horns. Virgin female Wistar rats (160 g) were injected with 0.2 mg of ß-estradiol in peritoneal 24 h before experiments. Uterus (2-3 cm) were vertically 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 $NaHCO_3$, 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 isotonic transducer (KN-259) and a recorder (KN-260) from Natsume Seisakusho, Japan.

Binding Experiments

Two rat uterus were homogenized with a Tokai micro-homogenizer M-100 in 12 ml of Munsick buffer containing 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin and 10 µg/ml leupeptin. To 1 ml of homogenate, 25 nCi of ^{125}I-endothelin and various calcium-channel antagonists were added in the presence and absence of 1 µM unlabeled endothelin. The mixture was incubated for 1 h at 37°C and then free ^{125}I-endothelin was moved by centrifugation. Radioactivity was measured with an Aloka gamma counter ARC-300.

RESULTS

Endothelin caused contractions of rat uterus in a dose-dependent manner (Fig. 1); its effects became apparent at 0.3 nM. This contractile potency of endothelin for uterus is similar to

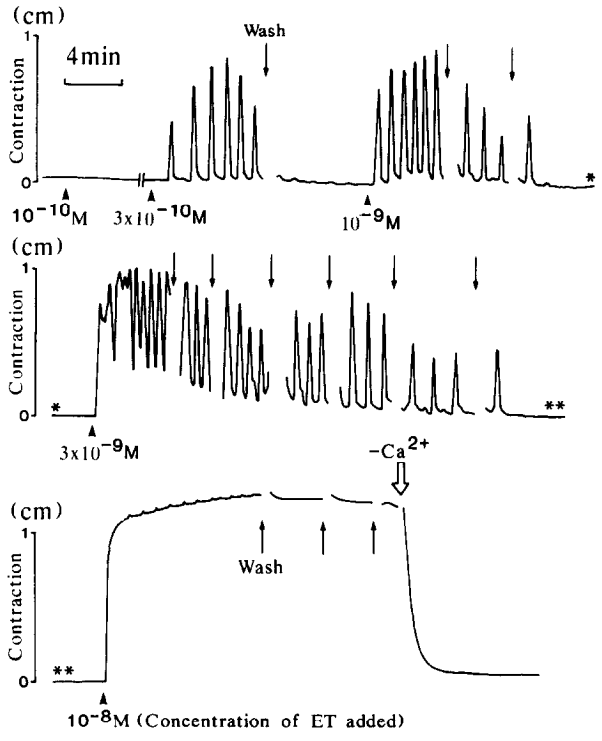

Figure 1. Typical constrictive responses of rat isolated uterus to various concentrations of endothelin. Endothelin (ET) was added at the point indicated by arrow heads (▲). The same uterine horns were repeatedly used in this experiment. ⟶:uterus was rinsed with Munsick buffer. ⇨:the solution in the bath was substituted with calcium-free Munsick buffer plus 1 mM EDTA.

that reported for coronary artery strip (1). The endothelin-induced uterine contractions were biphasic, namely they contained two components: rhythmic and developing contractions (see also Fig. 2b), the latter being predominant at higher concentrations of endothelin. When calcium was removed from the incubation medium, the contractile responses were completely lost, indicating that calcium is essential for endothelin's ability to contract uterine smooth muscle. The effect of endothelin was relatively difficult to wash out; even the contractions induced by a low concentration of endothelin (3 nM) required six changes of bathing medium to be completely washed out (Fig. 1). Figure 2 shows the effect of nifedipine on endothelin-induced uterine contractions. Nifedipine is a dihydropyridine antagonist that

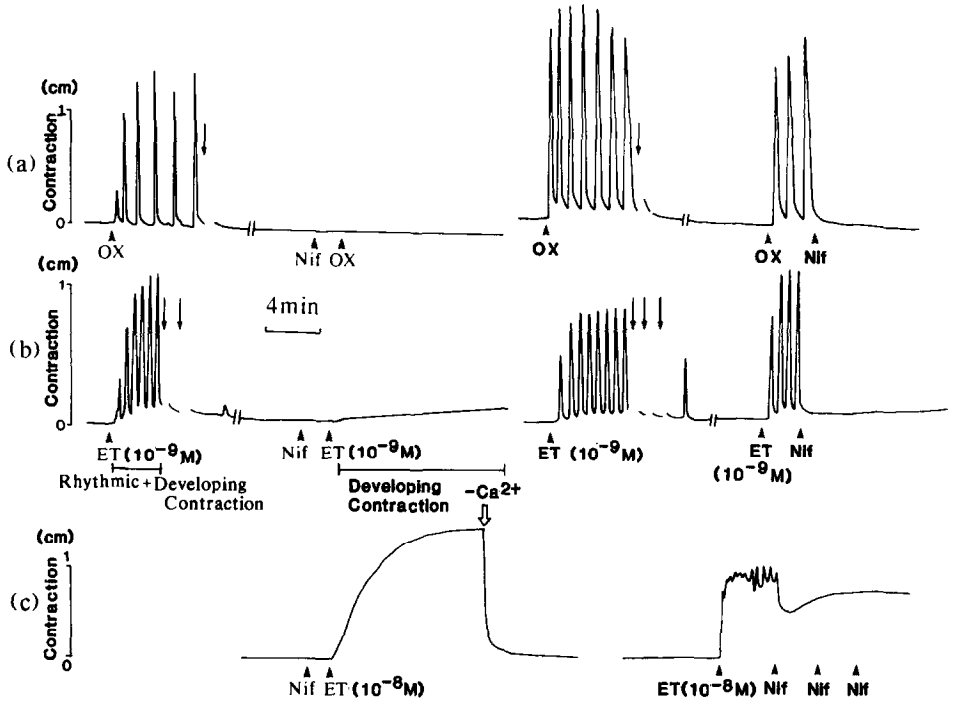

Figure 2. Inhibitory effects of nifedipine on uterus contraction by oxytocin and endothelin. Oxytocin (OX, 0.55 nM) and endothelin (ET, 10 nM and 1 nM) were added to the bath at the point indicated by arrow heads (▲). Experiments were carried out in the dark during the use of nifedipine(Nif, 100 nM). ⟶:uterus was rinsed with Munsick buffer. ⇨:the solution in the bath was substituted with calcium-free Munsick buffer plus 1 mM EDTA.

blocks the activity of voltage-dependent calcium channel. Addition of 0.1 μM nifedipine before or during endothelin treatment completely abolished the rhythmic contractions (Fig. 2b); however, the developing contractions remained unaffected (Figs. 2b and 2c). As a control, nifedipine-sensitive, oxytocin-induced contractions of uterine horns are shown in Fig. 2a. The other calcium channel antagonists nitrendipine (0.1 μM), verapamil (1 μM), and diltiazem (1 μM) also blocked the rhythmic contractions induced by endothelin and oxytocin. Similarities are remarkable between the endothelin-induced and oxytocin-induced rhythmic contractions of rat uterus.

TABLE 1

Binding of ^{125}I-endothelin to rat uterus membranes in the presence of various competitors

Competitor		^{125}I-Endothelin bound (cpm)	Bound/Total (%)
None		16,616	33.5
Endothelin	(1 μM)	6,830	13.8
Nifedipine	(0.1 μM)	17,379	35.0
Nitrendipine	(0.1 μM)	16,872	34.0
Verapamil	(1 μM)	15,859	32.0
Diltiazem	(1 μM)	14,664	29.6

To gain insight into the binding site of endothelin, we next carried out competitive binding assays using ^{125}I-endothelin. As shown in Table 1, no competition was observed between the calcium channel antagonists and endothelin.

DISCUSSION

Much controversy has been generated concerning the mechanism of action of endothelin on vascular smooth muscle. We are therefore interested in studying the mechanism using more simple systems, and have chosen to work with rat uterus since this organ has been shown to have unique contractile properties represented by rhythmic contractions (7-9). The present study demonstrated that endothelin, like oxytocin, causes rhythmic contractions of rat uterus, which are dependent on extracellular calcium and sensitive to voltage-dependent calcium channel blockers. Similarities between the actions of endothelin and oxytocin (Fig. 2) and the fact that the binding sites for endothelin are different from those for calcium channel antagonists (Table 1) strongly suggest that endothelin indirectly modulates the opening of voltage-dependent calcium channels.

Inositol trisphosphate is a strong candidate for the mediator that links the receptor site and the effector site (calcium channels). Recently, Marc et al. (10) demonstrated that

oxytocin stimulates a specific receptor-mediated phospholipase C activation which results in the accumulation of inositol phosphates and suggested that inositol trisphosphate may be considered as a potential intracellular messenger for the contractile effect elicited by oxytocin; furthermore, direct interaction of inositol trisphosphate with voltage-dependent calcium channels in the plasma membranes has been demonstrated by Vilven and Coronado (11). A similar mechanism may also underlie the action of endothelin.

The second type of contraction, namely developing contraction, is also an interesting response of uterus to endothelin. It is a new type of endothelin-induced contraction; its features include 1) slow onset, 2) insensitivity to calcium antagonists, and 3) absolute dependence on extracellular calcium. These characteristics indicate that the uterine smooth muscle has a second endothelin-sensitive pathway to regulate calcium influx.

The results reported here suggest that endothelin may play a role also in the regulation of nonvascular smooth muscle tone by way of multiple routes.

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REFERENCES

1. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature **332**, 411-415
2. Kopeyan, C., Martinez, G. and Rochat, H. (1985) FEBS Lett. **181**, 211-217
3. Habermann, E. (1972) Science **177**, 314-322
4. Kloog, Y., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. and Bdolah, A. (1988) Science **242**, 268-270
5. Auguet, M., Delaflotte, S., Chabrier, PE., Pirotzky, E., Clostre, F. and Braquet, P. (1988) Biochem. Biophys. Res. Commum. **156**, 186-192

6. Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T. X., Kumagai, S., Nakajima, K. and Sakakibara, S. (1988) Biochem. Biophys. Res. Commum. **154**, 868-875
7. Grover, A. K. and Oakes, P. J. (1985) Life Sci. **37**, 2187-2192
8. Granger, S. E., Hollingsworth, M. and Weston, A. H. (1985) Br. J. Pharmacol. **85**, 255-262
9. Leavitt, W. W., Burns, J. M. and Alecozay, A. (1987) "Peptide Hormone Receptor" (eds. Kalimi, M.Y. & Hubbard, J. R.) Walter de Gruyter & Co., Berlin, pp. 615-638
10. Marc. S., Leiber, D. and Harbon, S. (1988) Biochem. J. **255**, 705-713
11. Vilven, J. and Coronado, R. (1988) Nature **336**, 587-589