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### Research Article

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# Endothelin- and Oxytocin-induced Calcium Signaling in Cultured Human Myometrial Cells

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## Abstract

The demonstration that endothelin (ET) induces rat uterine contraction, coupled with the observation that ET is present in human amniotic fluid, suggests that the myometrium may be an important target organ for this hormone. We show that in quiescent human myometrial cells ET produced a dose-dependent increase in cytosolic free  $Ca^{2+}$  ( $Ca_i^{2+}$ ), which was markedly attenuated when the cells were studied in  $Ca^{2+}$ -free media. Preincubation with nicardipine, diltiazem, or verapamil reduced the ET-evoked  $Ca_i^{2+}$  transient by 30, 40, and 65%, respectively. The presence of voltage sensitive  $Ca^{2+}$  channels was demonstrated by  $Mn^{2+}$  quenching of fura-2. Activation of the  $Na^+/H^+$  antiport could not be demonstrated with ET stimulation. In nonquiescent cells, the ET-evoked  $Ca_i^{2+}$  transient was significantly reduced, while the response to oxytocin was retained. This is at least partially explained by a reduction in  $B_{max}$  (maximal binding capacity) for ET (mean  $\pm$  SEM) from  $3,506 \pm 268$  binding sites/cell in quiescent cells to  $2,411 \pm 300$  binding sites/cell, as well as a 72% increase in  $K_d$  (equilibrium dissociation constant), in the nonquiescent cells. We conclude that, in human myometrial cells, ET and oxytocin modulate  $Ca_i^{2+}$  through independent receptors and propose that ET, like oxytocin, is an important endogenous modulator of uterine contractility. (*J. Clin. Invest.* 1991. 87:1251–1258) Key words: myometrium • quiescence • smooth muscle • receptors •  $Mn^{2+}$  quenching •  $Ca^{2+}$  channels

## Introduction

The physiological and biological importance of endothelin (ET)<sup>1</sup> is supported by the demonstration that the peptide circulates in plasma (1), by its vasoconstrictive effect (2, 3), and by its enhancement of cell growth (4) and stimulation of *c-myc* and *c-fos* (5). Recently, it has been shown that ET is present in human amniotic fluid in concentrations significantly higher than in plasma (6). This finding and the observation that ET

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1. Abbreviations used in this paper:  $B_{max}$ , maximal binding capacity; BCECF-AM, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetyloxymethyl ester;  $Ca_i^{2+}$ , cytosolic free  $Ca^{2+}$ ; ET, endothelin; FBS, fetal bovine serum; HBS, Hepes buffered solution; pH<sub>i</sub>, cytosolic pH.

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induces rhythmic contractions in rat uterine horns (7) indicate that the myometrium may be an important target organ of ET. This investigation was undertaken to characterize the effects of ET, in comparison with oxytocin, in cultured human myometrial cells. We demonstrate that ET and oxytocin exert their effects on myometrial cells via binding to specific receptors and stimulation of the  $Ca^{2+}$  messenger system. These findings suggest that, in addition to oxytocin, ET plays an important role in the cellular homeostasis of the myometrium.

## Methods

Oxytocin, nicardipine, diltiazem, and verapamil were from Sigma Chemical Co. (St. Louis, MO). Synthetic endothelin (ET-1) was from Cambridge Biochemicals (Valley Stream, NY), and <sup>125</sup>I-ET was from Peninsula Laboratories (Belmont, CA). Fura-2AM and 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetyloxymethyl ester (BCECF-AM) were from Molecular Probes (Eugene, OR). All cell culture materials were from Hazleton Biologics, Inc. (Kansas City, MO). Immunofluorescence antibodies were from Organon Teknika Corp. (West Chester, PA).

**Cell culture.** Uterine tissue (~ 0.5 g per specimen) originated from 10 patients during cesarean section under epidural or general anesthesia. Gestational age ranged from 37–40 wk. 8 patients had low segment transverse incision and 2 had classical midline incision. The tissue was obtained from the incision edge.

Myometrial tissue was placed in 10 ml  $\alpha$ -MEM containing penicillin (30 U)/streptomycin (30  $\mu$ g/ml) (3 $\times$  MEM). The sample was rinsed three times with 3 $\times$  MEM and the tissue finely chopped. Cell cultures from each patient were initiated using two different methods, namely, cell suspension and tissue explant. For the cell suspension method, the tissue was transferred to a 15-ml conical test tube containing 10 ml of 3 $\times$  MEM and centrifuged for 8 min at 1000 g. Supernatant was aspirated and the pellet rinsed and spun twice with  $\alpha$ MEM plus 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin (10 U/ml)/streptomycin (10  $\mu$ g/ml). The final 10 ml was split among three 75-cm<sup>2</sup> Nunc flasks and brought to 15 ml with  $\alpha$ MEM. Cells were grown in 5% CO<sub>2</sub> at 37°C and refed twice each week.

For the explant method, tissue fragments were placed directly into 75-cm<sup>2</sup> flasks using a pasteur pipette. Flasks were inverted in the incubator at 5% CO<sub>2</sub>, 37°C for 1 h to allow cell attachment. 15 ml of  $\alpha$ MEM were added slowly to avoid disturbing the fragments and flasks placed in the incubator with the fragments on the bottom. Cultures were maintained as per the cell suspension method. There were no effects of the method of cell isolation and passage number on cellular signals. Nevertheless, cells from identical passages isolated by the same method, from at least four subjects, were used in each experiment presented in Figs. 2–9. Experiments were performed using passages 1–6.

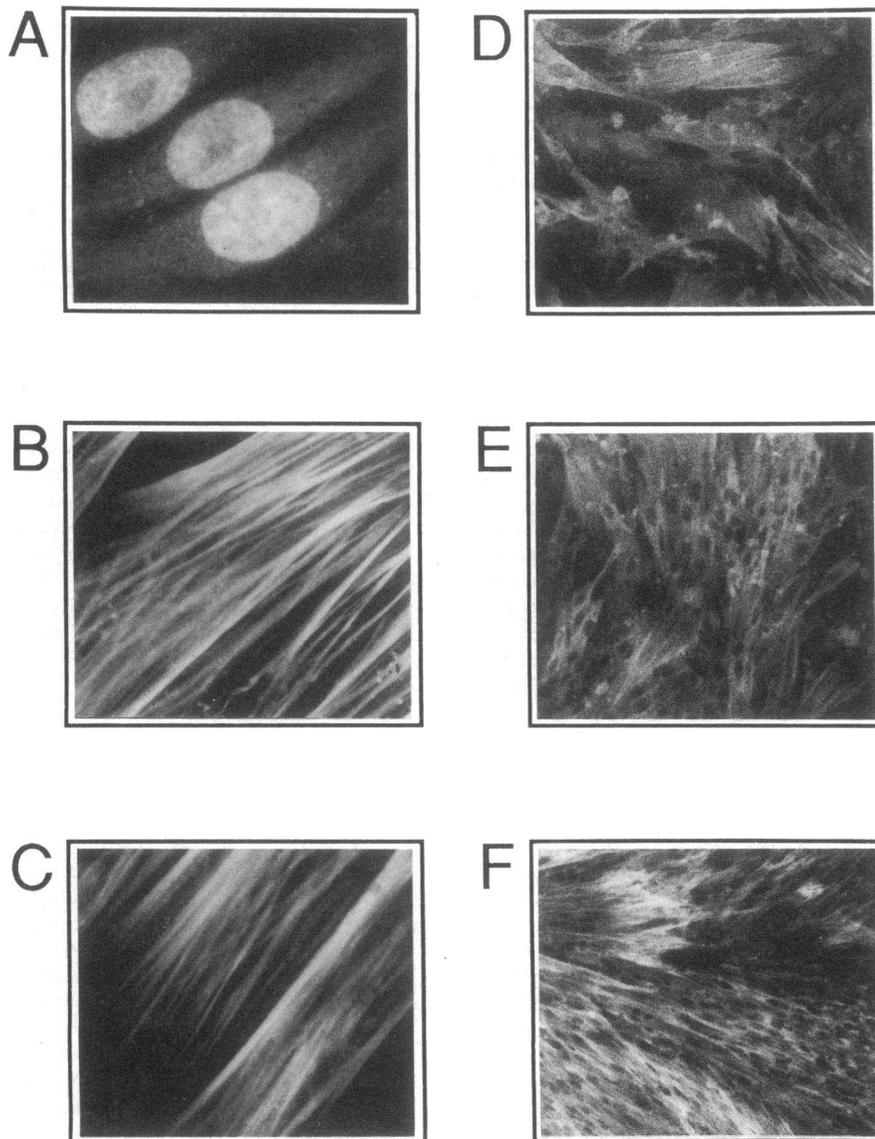
**Cytosolic free  $Ca^{2+}$  ( $Ca_i^{2+}$ ) and cytosolic pH (pH<sub>i</sub>) measurements.** To measure  $Ca_i^{2+}$ , coverslips containing 3–7  $\times$  10<sup>5</sup> cells were incubated with 5  $\mu$ M fura 2AM in 2 ml (37°C) of Hepes buffered solution (HBS) containing (mM): NaCl, 140; KCl, 5; CaCl<sub>2</sub>, 2; MgCl<sub>2</sub>, 1; Hepes, 10; glucose, 10; and 0.1% BSA, pH 7.4. Cells were loaded for 60 min, washed three times with HBS, and secured in a quartz cuvette in a SPEX CM3 fluorescence spectrometer (Spex Industries, Inc., Edison,

NJ) (equipped with a thermostatically controlled (37°C) cell holder, stirrer, and suction device for rapidly removing solutions). Excitation wavelengths were set at 340 and 380 nm and emission wavelength at 505 nm. Cells were preincubated 1–3 min in HBS until the signal stabilized. In experiments performed in Ca-free medium, CaCl<sub>2</sub> was omitted and 0.3 mM EGTA added to the HBS. At the end of each experiment autofluorescence was determined for each coverslip (by subjecting cells to 2 mM Mn<sup>2+</sup> and 10 μM ionomycin for 5 min) and subtracted from the fluorescent signal. Calculations of  $R_{max}$  and  $R_{min}$  and  $S_{F2}/S_{b2}$  (8) for Ca<sup>2+</sup> calibration were performed on similarly prepared coverslips by exposing cells to 3 mM EGTA, 10 μM ionomycin (without and with 0.1% BSA), and 5 mM Ca<sup>2+</sup>, 10 μM ionomycin (without and with 0.1% BSA).

To measure pH<sub>i</sub>, coverslips were incubated with 5 μM BCECF-AM at 37°C in HBS for 1 h and washed twice with HBS. Fluorescence was monitored (excitation wavelengths 440 and 503 and emission wavelength 530 nm) for 5–7 min in HBS (pH 7.4) until pH<sub>i</sub> stabilized. Calibration of pH<sub>i</sub> was performed as previously described (9) by subjecting cells to 5 μg/ml nigericin in HBS (minus BSA) at pH values ranging between 6.4 and 7.6, and K<sup>+</sup> of 140 mM (KCl substituted isosmotically for NaCl). For experiments in which NaCl was less than 140 mM, *N*-methyl-D-glucamine was substituted isosmotically for Na<sup>+</sup>.

**Mn<sup>2+</sup> uptake.** For these experiments, cells were treated as per measurements of Ca<sub>i</sub><sup>2+</sup>. They were suspended in HBS plus 1 mM MnCl<sub>2</sub>. Mn<sup>2+</sup> uptake by the cells was monitored at 360 nm. This is the isosbestic wavelength for fura-2. Thus, changes in Ca<sub>i</sub><sup>2+</sup> do not alter the fura-2 emission, whereas Mn<sup>2+</sup> uptake results in quenching the fluorescence.

**<sup>125</sup>I-ET binding experiments.** Cells were inoculated in Nunc 24-well plates, grown for 3 d in 10% FBS and serum deprived for 24 h to reach quiescence. To study nonquiescent cells on the same day at approximately the same cell density, cells were inoculated at a lower density and grown for 4 d. In preliminary experiments, specific ET binding at 22°C reached a plateau within 120 min and remained stable for at least 240 min. For all further experiments, cells were incubated for 150 min at 22°C in 1 ml HBS containing 1.8 mM Ca<sup>2+</sup>, 0.2% BSA, 100 KU/ml aprotinin, 31.2 pM <sup>125</sup>I-ET (sp act = 1,564 Ci/mmol), and varying concentrations (0.03–50 nM) of unlabeled ET. Oxytocin (10<sup>-7</sup> M) and aliquots of serum or αMEM were added to the medium in studies examining their effect on ET binding. After incubation, the medium was aspirated and cells were washed 4 times with 2 ml ice-cold HBS. Cells were extracted with 5% TCA and bound <sup>125</sup>I-ET measured in a gamma counter. Total binding in quiescent cells averaged (mean±SEM) 0.17±0.01% of total activity added to each well, and nonspecific binding (determined in the presence of 400 nM unlabeled ET) averaged 15.2±0.34% of total binding. Total binding in nonquie-



**Figure 1.** Immunofluorescence using HNF35 muscle actin antibody. (A) Human skin fibroblasts; (B) human vascular smooth muscle cells; (C) human myometrial cells; and (D, E, and F) fields of myometrial cells at various stages of confluency. “Stress fibers” are seen only in myometrial and vascular smooth muscle cells. Magnification for A–C is at 1,250; for D at 150; and for E and F at 100.

scent cells averaged  $0.11 \pm 0.01\%$  of total activity added to each well and nonspecific binding was  $13.5 \pm 0.28\%$  of total binding.

**Immunofluorescence.** HHF35, a muscle-actin-specific monoclonal antibody, was used to ascertain purity and composition of the myometrial smooth muscle cell preparations. Immunofluorescence was measured as described previously (10). There were no differences in immunofluorescence characteristics of cells from the various passages prepared by the cell suspension or the tissue explant methods. 20 fields of cells from passages 1–4 from 4 subjects were examined. No apparent contaminations with fibroblasts of the preparations were observed.

**Data analysis.** Data are presented as mean or mean  $\pm$  SEM, with  $n$  equal to the number of individuals studied. Nonlinear regression analyses of ET binding experiments and  $Mn^{2+}$  quenching of fura-2 were performed on an IBM compatible personal computer using an NLIN (nonlinear) regression procedure of SAS (Statistical Analytic Systems) (11). Statistical analyses of these data utilized weighted least squares according to the method described by Johnson and Milliken (12).

## Results

Fig. 1 shows the immunofluorescence staining with the muscle-actin-specific antibody of human skin fibroblasts, human umbilical artery vascular smooth muscle cells, and human myometrial cells. Myometrial cells and vascular smooth muscle cells exhibit "stress fibers" transversing their longitudinal

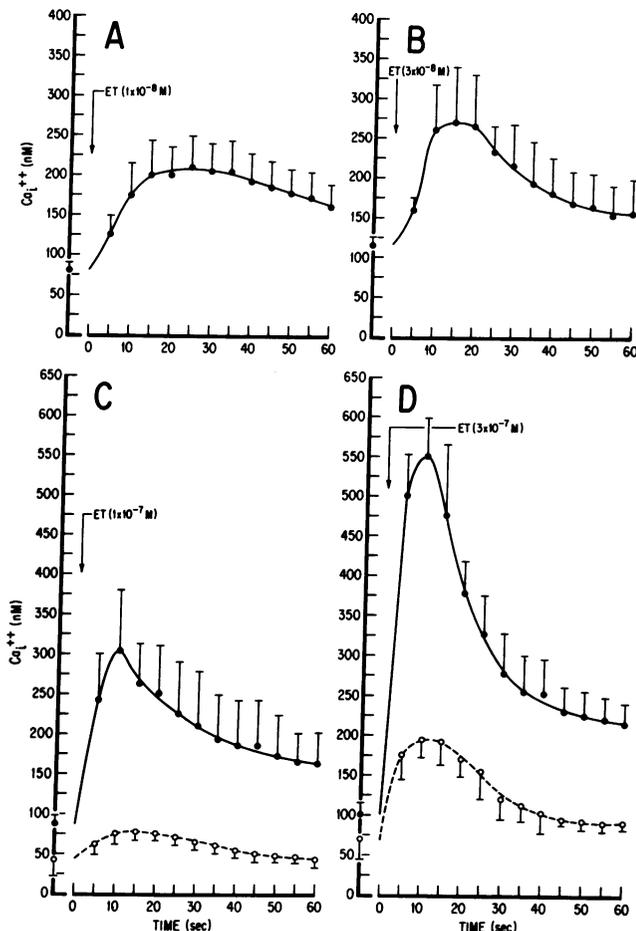


Figure 2. ET-dependent  $Ca_i^{2+}$  profiles. Closed symbols indicate HBS containing 1.8 mM  $Ca^{2+}$  ( $n = 7$  for each dose). Open circles indicate  $Ca^{2+}$ -free media ( $n = 5$  for each dose).

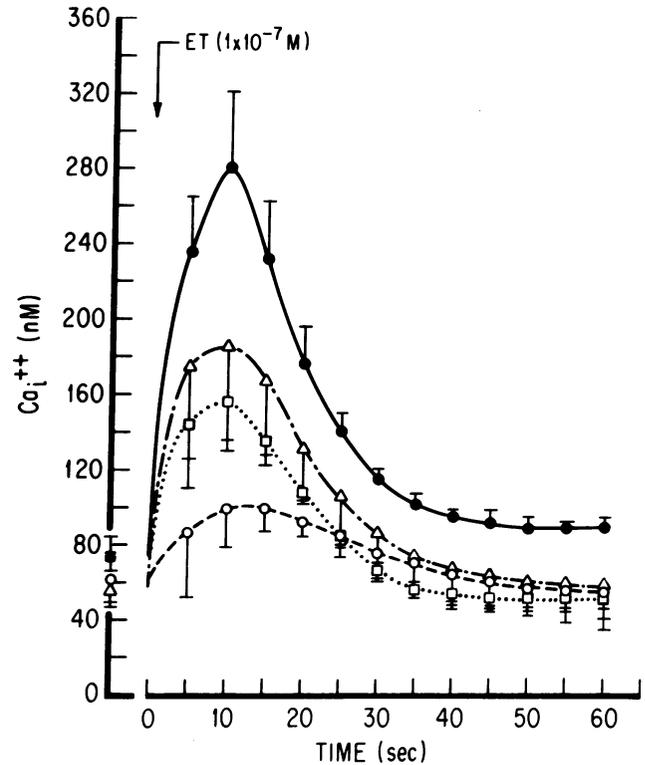


Figure 3. Effect of 10-min preincubation with  $Ca^{2+}$ -channel blockers on the ET-induced  $Ca^{2+}$  transients and posttransients. Closed circles indicate ET ( $n = 5$ ); open triangles indicate ET + nicardipine (0.3  $\mu$ M,  $n = 4$ ); open squares indicate ET + diltiazem (10  $\mu$ M,  $n = 4$ ); open circles indicate ET + verapamil (10  $\mu$ M,  $n = 5$ ).

axis. This is a typical presentation of smooth muscle actin (10, 14).

**Effects of ET and Oxytocin on  $[Ca^{2+}]_i$ .** Myometrial cells deprived of serum for 24 h and equilibrated in HBS containing 1.8 mM  $Ca^{2+}$  had basal  $[Ca^{2+}]_i$  of  $102.5 \pm 3.6$  nM. Basal  $[Ca^{2+}]_i$  was reduced by 43% to  $58.5 \pm 2.9$  nM when the cells were equilibrated for 3 min in  $Ca^{2+}$ -free HBS.

Addition of ET induced a dose-dependent increase in  $[Ca^{2+}]_i$  with peak levels ranging from  $211.7 \pm 38.5$  nM at a dose of  $1 \times 10^{-8}$  M to  $583.0 \pm 50.5$  nM at a dose of  $3 \times 10^{-7}$  M (Fig. 2). The response to ET was markedly reduced when the cells were studied in  $Ca^{2+}$ -free media (Fig. 2, C and D), demonstrating that the ET-induced  $Ca_i^{2+}$  signal in the myometrial cells is also dependent on extracellular  $Ca^{2+}$ . To examine if this dependency reflects influx through  $Ca^{2+}$  channels, cells were preincubated for 10 min with various channel blockers before stimulation by ET (Fig. 3). Preincubation with nicardipine (0.3  $\mu$ M), diltiazem (10  $\mu$ M), or verapamil (15  $\mu$ M) reduced the peak  $[Ca^{2+}]_i$  by 30, 40, and 65%, respectively, and reduced the post-transient response by  $\sim 40\%$  as compared with control. While these results are consistent with the observation that uterine contractions induced by ET are blocked by EGTA and  $Ca^{2+}$  channel antagonists (7), the partial dependence of ET-induced  $Ca_i^{2+}$  transients on extracellular  $Ca^{2+}$  in the myometrial cells suggests that ET mobilizes  $Ca^{2+}$  from intracellular stores as well.

Of interest was the observation that the depolarization of the myometrial cells by KCl (45 mM, replacing equimolarly NaCl) neither affected the  $Ca_i^{2+}$  signal nor altered the ET-in-

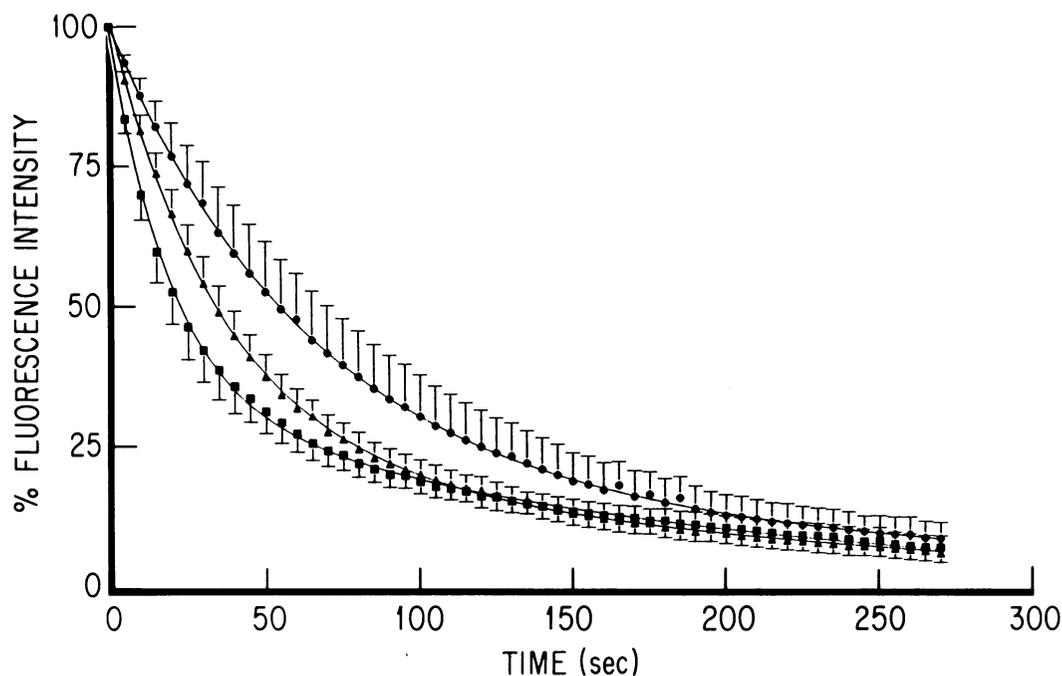


Figure 4.  $Mn^{2+}$  quenching of fura-2. Closed circles, control; closed triangles, 100 nM ET; closed squares, 45 mM KCl. Treatments with ET and KCl were performed at  $t = 0$  by replacing HBS with either KCl or ET HBS with 1 mM  $Mn^{2+}$ . Curves were fit to the data according to the following exponential model:

$$A = a_1 \times e^{-k_1 t} + a_2 \times e^{-k_2 t}$$

where:  $A$  = the cellular pool at time  $t$ ,  $a_{1,2}$  are compartments (expressed as percentage of the cellular pool; i.e., fura-2 fluorescence before treatment with ET or KCl) and  $k_{1,2}$  are rate constants. For further details, see Table I.

duced  $Ca_i^{2+}$  response (not shown). This observation appears inconsistent with the findings that L-type  $Ca^{2+}$  channel blockers attenuated the ET-evoked  $Ca_i^{2+}$  response. To address this issue, further experiments were performed using  $Mn^{2+}$  as a  $Ca^{2+}$  surrogate. In these experiments we monitored  $Mn^{2+}$  entry by the quenching of fura-2.

The profile of  $Mn^{2+}$  quenching of cellular fura-2 was consistent with two compartments ( $a_1$  and  $a_2$ ), which under control conditions, respectively, comprised  $\sim 79$  and 21% of the entire cellular pool (Fig. 4, Table I). Treatment with either 100 nM ET or 45 mM KCl resulted in a small reduction in  $a_1$  with a concurrent increase in  $a_2$  and a substantial increase in the rate constant ( $k_1$ ) of fura-2 quenching of  $a_1$ . There was also an increase in the rate of fura-2 quenching of  $a_2$ . The increase in  $k_1$  after KCl depolarization was, in fact, greater than that resulting from treatment with ET.

Oxytocin produced a dose-dependent increase in  $[Ca^{2+}]_i$  (Fig. 5), reaching a similar peak  $[Ca^{2+}]_i$  at a dose of 100 nM ( $292.2 \pm 12.4$  nM) as that for the same dose of ET ( $304.5 \pm 78.7$  nM). Both ET and oxytocin demonstrated desensitization in quiescent cells. As shown in Fig. 6, 3 min after addition of agonist and several washings with HBS, a second  $Ca_i^{2+}$  transient could not be evoked with addition of the same agonist.

However, when ET and oxytocin were added sequentially, each agonist produced a normal  $Ca_i^{2+}$  transient (Figs. 7 and 8). Thus, ET and oxytocin produce  $Ca_i^{2+}$  transients through distinct receptors. Further differentiating the action of the two hormones is the observation that, in nonquiescent cells, the response to ET was significantly reduced while the response to the sequential treatment with oxytocin in the same cells was retained (Fig. 7 B). When the order of addition of agonist was reversed, namely, oxytocin was added first, ET, again, had little effect in nonquiescent cells (Fig. 8 B). The unaltered oxytocin-induced  $Ca_i^{2+}$  response in the nonquiescent cells indicates that serum-induced desensitization to ET does not represent a cellular dysfunction.

Many agonists elevate  $Ca_i^{2+}$  in concert with stimulation of the  $Na^+/H^+$  antiport (13). To determine if ET can activate the  $Na^+/H^+$  antiport in these cells, the peptide was added under three experimental conditions. Firstly, addition of agonist to cells at basal  $pH_i$  ( $6.96 \pm 0.06$ ) was without effect over a 10-min period (Fig. 9 A). Secondly, in cells in which the antiport was reversed by addition of  $Na^+$ -free media, ET failed to further stimulate the antiport (Fig. 9 B). Although not shown, reversal of the pH gradient by incubating the cells in  $Na^+$ -free buffer at pH 6.45 did not facilitate activation of the antiport by ET. In

Table I. Quenching of Fura-2 by Mn

Treatment	$k_1^*$	$a_1^*$	$k_2^*$	$a_2^*$
	$s^{-1}$	%	$s^{-1}$	%
Control	$0.0162 \pm 0.0002$	$78.88 \pm 0.86$	$0.0036 \pm 0.0001$	$21.43 \pm 0.90$
100 $\mu$ M ET	$0.0293 \pm 0.0002$	$74.96 \pm 0.44$	$0.0050 \pm 0.0001$	$26.76 \pm 0.48$
45 mM KCl	$0.0513 \pm 0.0005^\ddagger$	$65.49 \pm 0.31$	$0.0057 \pm 0.0001$	$33.65 \pm 0.28$

$a_{1,2}$  = Compartments (expressed as percentage of the cellular pool);  $k_{1,2}$  = rate constants,  $t$  = time ( $n = 6$  for each treatment). Statistical analyses were performed using the Johnson and Milliken procedure, as indicated under Methods. \* Both KCl and ET treatments significantly differ from control at  $P < 0.001$ .  $^\ddagger$  Significantly different from ET treatment at  $P < 0.001$ .

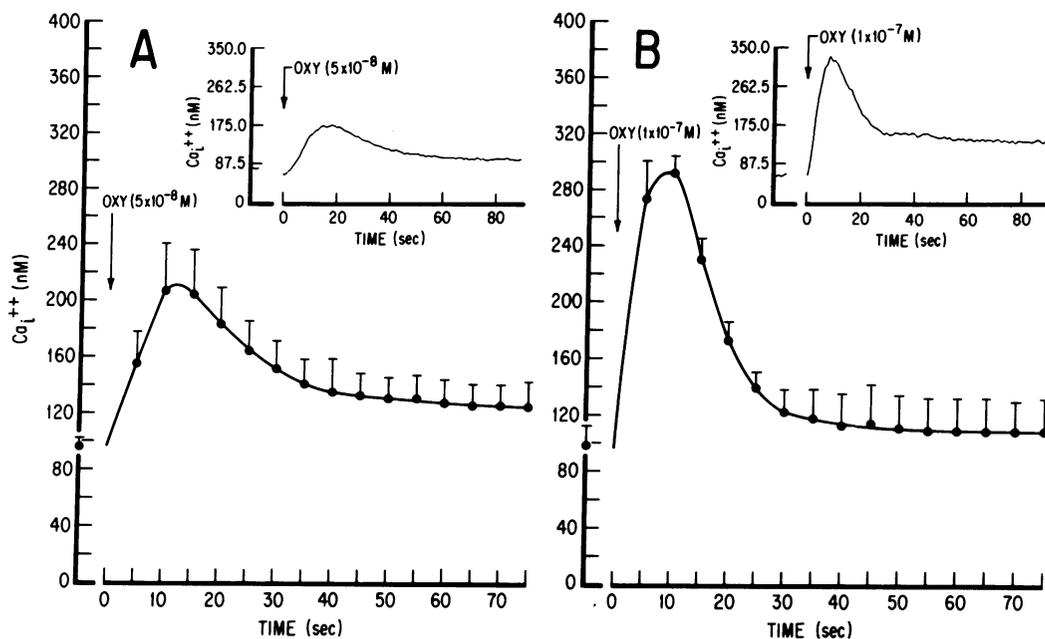


Figure 5.  $Ca_i^{2+}$  profiles elicited by oxytocin. (A) 50 nM ( $n = 5$ ); (B) 100 nM ( $n = 4$ ). Insets show typical transients at each dose.

the third experiment, following cellular acidification by the ammonium chloride prepulse method, the addition of ET to HBS containing 40 mM  $Na^+$  did not accelerate the  $Na^+$ -dependent recovery (the  $Na^+/H^+$  antiport) (Fig. 9 C). Thus, although the  $Na^+/H^+$  antiport is present in myometrial cells, there is no apparent stimulation of this system by ET.

*ET receptor binding experiments.*  $^{125}I$ -ET binding to myometrial cells at 22°C showed saturability, reaching its plateau by 2 h. The following data were obtained from binding experiments depicted in Fig. 10. Quiescent cells showed a  $B_{max}$  of  $3,506 \pm 268$  binding sites/cell and  $K_d$  of  $125 \pm 15$  pM. The Hill coefficient was 0.99, indicating no evidence of cooperativity in

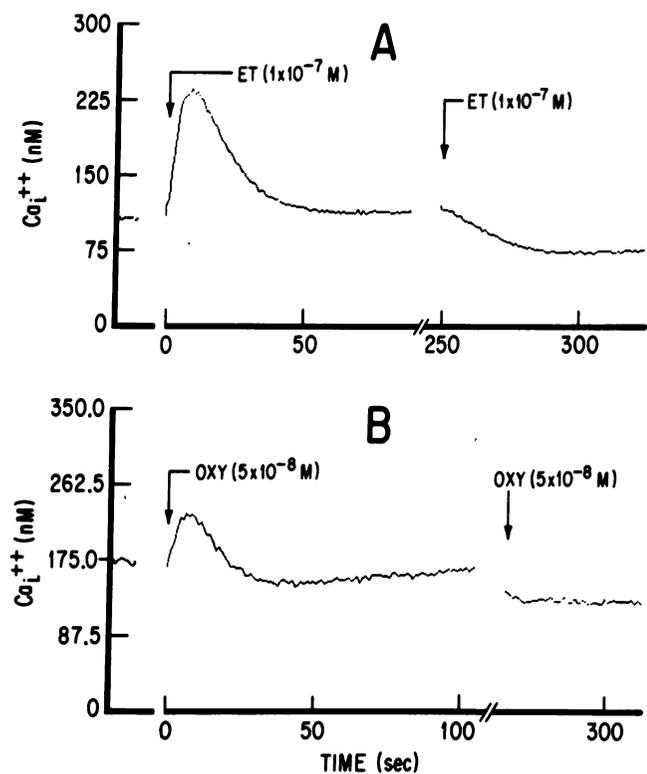


Figure 6. Representative tracings of  $Ca_i^{2+}$  transients evoked by (A) ET 100 nM and (B) oxytocin 100 nM. 3 min after the first transient (and three washes with HBS) cells were stimulated again. The decline in the  $Ca^{2+}$  signal after washings probably represents loss of some extracellular fluorescence due to dye leakage.

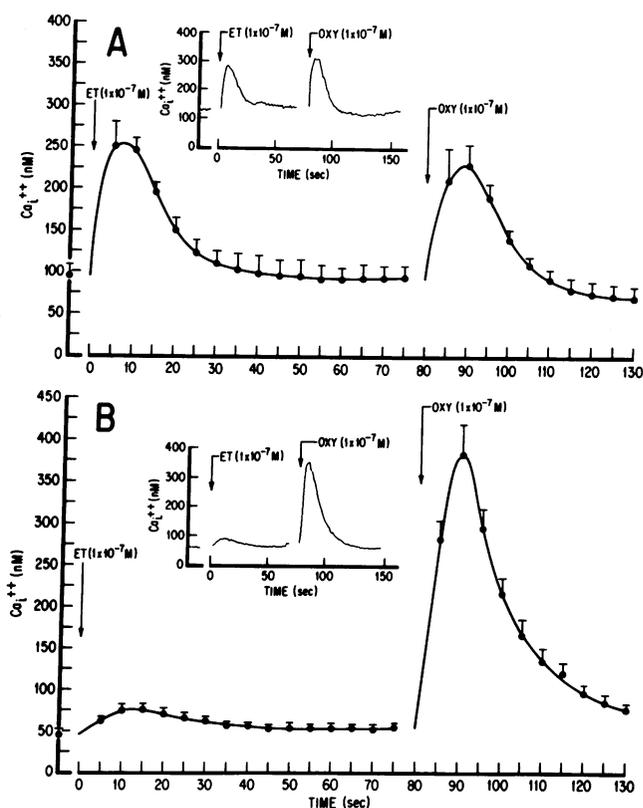


Figure 7.  $Ca_i^{2+}$  profile elicited by addition of ET 100 nM followed by oxytocin 100 nM in (A) quiescent cells ( $n = 4$ ) and (B) nonquiescent cells ( $n = 4$ ). Insets are representative tracings.

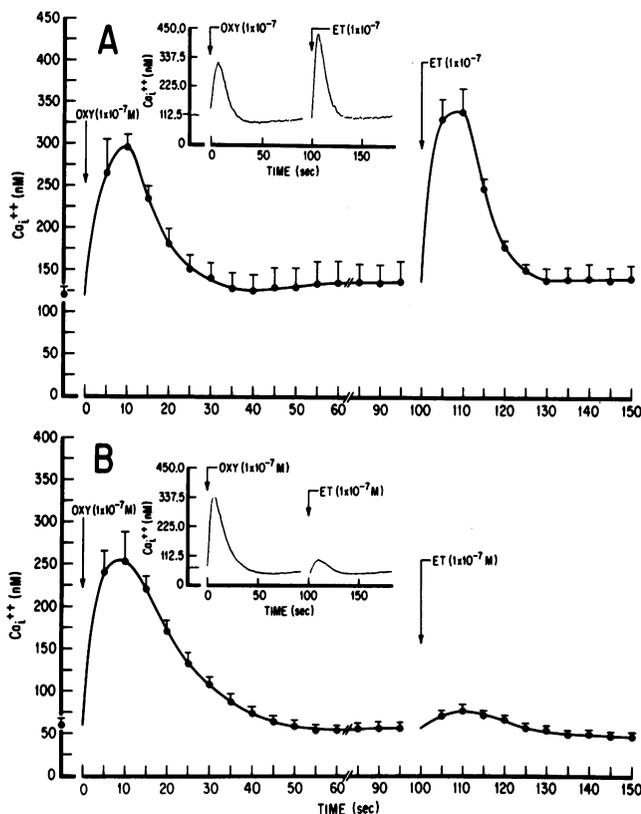


Figure 8.  $\text{Ca}_i^{2+}$  profile elicited by addition of oxytocin 100 nM followed by addition of ET 100 nM in (A) quiescent cells ( $n = 4$ ) and (B) nonquiescent cells ( $n = 5$ ). Insets are representative tracings.

ET binding to myometrial cells.  $B_{\max}$ ,  $K_d$ , and the Hill coefficient were not significantly changed by the addition of a fixed concentration of oxytocin (100 nM), indicating different receptors for ET and oxytocin. Nonquiescent cells demonstrated a significantly lower  $B_{\max}$  ( $2,411 \pm 300$  binding sites/cell) ( $P < 0.05$ ) and higher  $K_d$  ( $216 \pm 39$  pM) ( $P < 0.05$ ) than the quiescent cells. Although not shown,  $B_{\max}$  and  $K_d$  were not altered by the addition of 10% FBS or 250  $\mu\text{l}$  of conditioned  $\alpha\text{MEM}$  (obtained after 48 h incubation with myometrial cells).

## Discussion

The mechanism by which ET raises  $\text{Ca}_i^{2+}$  is as yet undefined and, in fact, may differ depending on the cell under investigation. The ET-induced  $\text{Ca}_i^{2+}$  signal in the myometrial cells is in part due to  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$  channels. This is shown by the reduction in both transient and posttransient  $\text{Ca}_i^{2+}$  with  $\text{Ca}^{2+}$  channel inhibitors. Similar conclusions were obtained for the action of ET on vascular smooth muscle cells (5) and fibroblasts (4). It is clear from several studies that the ET receptor is distinct from these  $\text{Ca}^{2+}$  channels (7, 14, 15) and, thus, the ET-dependent activation of  $\text{Ca}^{2+}$  channels must be a consequence of processes distal to the interaction of ET with its specific receptors. The dependence on extracellular  $\text{Ca}^{2+}$ , however, is not an absolute requirement of ET action, since a rise in  $\text{Ca}_i^{2+}$  has been demonstrated in the absence of  $\text{Ca}^{2+}$  entry through  $\text{Ca}^{2+}$  channels in fibroblasts and vascular smooth muscle cells (15, 16).

In this study we could demonstrate the presence of voltage sensitive  $\text{Ca}^{2+}$  channels in human myometrial cells by  $\text{Mn}^{2+}$  quenching of fura-2. This approach has been used before to characterize cellular  $\text{Ca}^{2+}$  regulation in a variety of cells (17–21).  $\text{Mn}^{2+}$  has a substantially greater affinity to fura-2 than  $\text{Ca}^{2+}$ , and it is a poor substrate for the  $\text{Ca}^{2+}$ -pump of the endoplasmic reticulum (21, 22). The biological equivalents of compartments  $a_1$  and  $a_2$  are not certain. Since compartment  $a_1$  exhibited rapid quenching under control condition and after stimulation by KCl and ET, it might represent the submembrane domain. Compartment  $a_2$  would represent a more central portion of the cytosol and cellular organelles accessible to fura-2 and  $\text{Mn}^{2+}$  (probably not the endoplasmic reticulum).

We propose that the activity of voltage-sensitive  $\text{Ca}^{2+}$  chan-

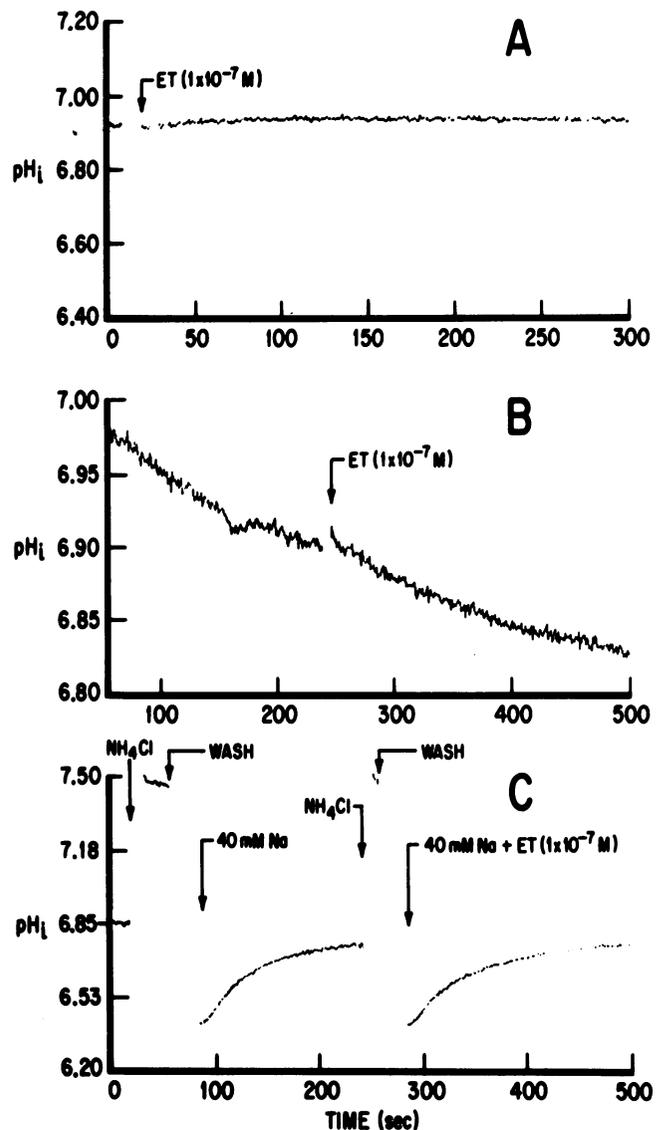


Figure 9. Effects of ET on  $\text{pH}_i$ . (A) ET added at basal  $\text{pH}_i$ . (B) Cells acidified in  $\text{Na}^+$ -free buffer and stimulated with ET after 4 min. (C) Effect of ET on  $\text{Na}^+$ -dependent recovery of  $\text{pH}_i$  in HBS containing 40 mM  $\text{Na}^+$  after acidification by an  $\text{NH}_4\text{Cl}$  prepulse. Cells were washed twice in  $\text{Na}^+$ -free HBS before addition of  $\text{Na}^+$ -containing HBS or HBS plus ET (100 nM). Tracings are representative of seven to nine observations for each experimental maneuver.

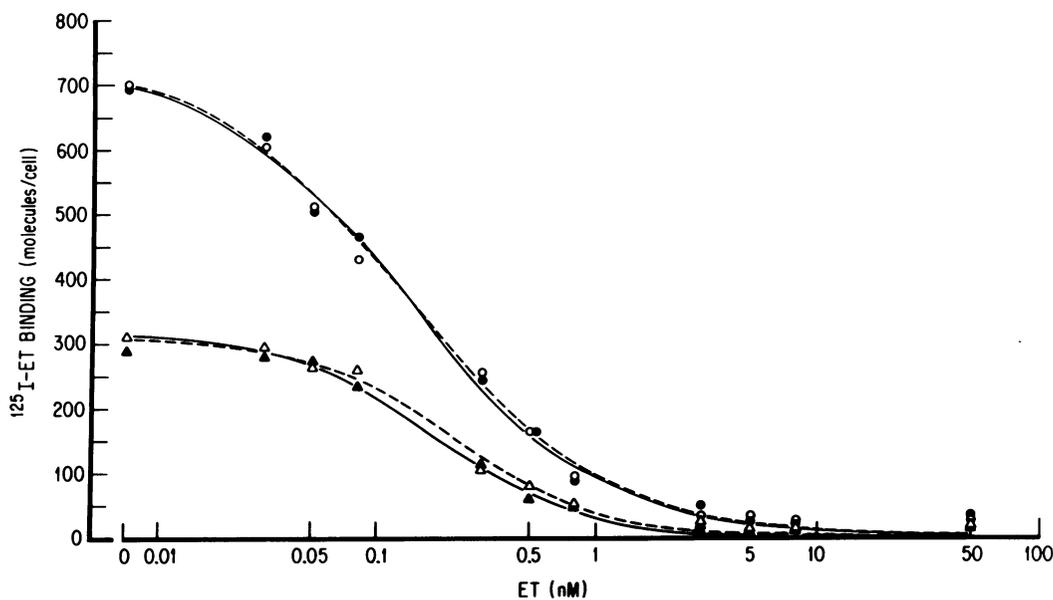


Figure 10. Displacement of  $^{125}\text{I}$ -ET by unlabeled ET in quiescent cells (circles) and nonquiescent cells (triangles). In quiescent cells, displacement of  $^{125}\text{I}$ -ET by unlabeled ET = closed circles, solid line ( $n = 4$ ), and unlabeled ET + oxytocin 100 nM = open circles, broken line ( $n = 4$ ). In nonquiescent cells, displacement of  $^{125}\text{I}$ -ET by unlabeled ET = closed triangles, solid line ( $n = 4$ ), and ET + oxytocin 100 nM = open triangles, broken line ( $n = 4$ ). Curves depict the fit of the model described by the following equation to the data:

$$B = B_{\max} \left\{ \frac{L}{K_d} \right. \\ \left. \times [1 + (i/K_d)^N] + L \right\}$$

where  $B$  is the specific binding,  $B_{\max}$  is the maximal specific binding,  $K_d$  is the equilibrium dissociation constant,  $L$  is the concentration of  $^{125}\text{I}$ -ET,  $i$  is the concentration of unlabeled ET, and  $N$  is the Hill coefficient.

nels in human myometrial cells is relatively low under basal state. KCl depolarization, thus, produced a relatively small acceleration in  $\text{Ca}^{2+}$  influx, insufficient for an apparent increase in the  $\text{Ca}_i^{2+}$ , probably because of recycling of the entering  $\text{Ca}^{2+}$  into the medium or its uptake by the endoplasmic reticulum. However, because of the above mentioned features of  $\text{Mn}^{2+}$ , its accelerated entry could be easily observed by fura-2 quenching. Since nifedipine, diltiazem, and verapamil attenuated the ET-evoked  $\text{Ca}_i^{2+}$  response, it is very likely that the voltage-sensitive  $\text{Ca}^{2+}$  channels are more active after treatment with ET, perhaps to refill cellular  $\text{Ca}^{2+}$  stores after  $\text{Ca}^{2+}$  mobilization.

Examination of the interaction between ET and oxytocin in this study revealed that both agonists act through distinct receptors. This was demonstrated both by the  $\text{Ca}_i^{2+}$  transient responses and the binding experiments which showed the lack of competition by oxytocin for the ET receptor.

An interesting difference between the actions of ET and oxytocin relates to the response of quiescent and nonquiescent myometrial cells to the two peptides. Oxytocin produced similar  $\text{Ca}_i^{2+}$  transients in quiescent and nonquiescent cells, while the ET-evoked transients were markedly blunted in nonquiescent cells. This phenomenon may not be unique to the myometrial cells, since it has been reported that ET produces a more consistent and potent  $\text{Ca}_i^{2+}$  response in mesangial cells after 72 h of serum deprivation (23). A partial explanation for the differences in ET-induced  $\text{Ca}_i^{2+}$  transients between quiescent and nonquiescent myometrial cells is provided by the binding experiments showing 31% fewer receptors per unit cell as well as a 72% decrease in the affinity of the receptor in the nonquiescent cells. This was not due to the presence of ET or an ET-like factor in FBS or conditioned  $\alpha\text{MEM}$ , since neither 10% FBS nor the conditioned medium altered the binding kinetics in quiescent cells. These data, thus, suggest that the coupling of the ET receptor to cellular pathways is also altered in the nonquiescent state.

Agonist-mediated stimulation of  $\text{Ca}_i^{2+}$  mobilization is often coupled to protein kinase C, an important modulator of the  $\text{Na}^+/\text{H}^+$  antiport (13). ET has been shown to elevate  $\text{pH}_i$  from basal levels in cultured mesangial cells (23) and to increase the rate of  $\text{Na}^+$ -dependent alkalization in human skin fibroblasts (24). However, ET had no effect on the  $\text{Na}^+/\text{H}^+$  antiport in the human myometrial cells, suggesting either that ET dissociates protein kinase C activation from activation of the antiport, or that the mechanism of ET-mediated  $\text{Ca}^{2+}$  mobilization is independent of protein kinase C. We arrived at a similar conclusion with respect to the effect of ET on the human umbilical artery vascular smooth muscle cell (unpublished data).

In conclusion, this study demonstrates that ET binds to a specific high affinity receptor on human myometrial cells, resulting in a rapid increase of  $\text{Ca}_i^{2+}$  by a mechanism involving mobilization of  $\text{Ca}^{2+}$  from intracellular stores, as well as  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$  channels. Moreover, ET and oxytocin modulate  $\text{Ca}_i^{2+}$  through independent receptors. These findings, and the recent observation that ET is produced by rabbit endometrial cells in culture (25) and is elevated in human amniotic fluid (6), strongly suggest that ET, like oxytocin, is an important endogenous modulator of uterine function.

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