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Novel Endothelin Receptors in the Follicular Membranes of *Xenopus laevis* Oocytes Mediate Calcium Responses by Signal Transduction through Gap Junctions

CHANDRIKA S. KUMAR, PARVATHI NUTHULAGANTI, MARK PULLEN, and PONNAL NAMBI

Departments of Molecular Genetics (C.S.K., P.N.) and Pharmacology (M.P., P.N.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406–0939

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SUMMARY

Follicular oocytes from *Xenopus laevis* display saturable and high affinity endothelin (ET)-1 binding sites. Competition binding experiments using unlabeled ET-1, ET-3, and sarafotoxin 6c indicated that these ET receptors belong to the ET_A receptor subtype. However, the ET_A receptor-selective antagonist BQ123 [cyclo(D-Trp,D-Asp,L-Pro,D-Val,L-Leu)] failed to inhibit ^{125}I-ET-1 binding to these receptors, suggesting that these receptors belong to a novel subtype of ET_A receptors (ET_{AX}) distinct from the human ET_A receptors. These endogenous receptors are present in the follicular layer, because defolliculated oocytes did not show any ^{125}I-ET-1 binding. Addition of ET-1 to follicular oocytes led to Ca^{2+} mobilization, which was reversibly blocked by treatments that uncouple gap junctions, suggesting that these ET_{AX} receptors mediate their response by transferring signals through gap junctions. On the other hand, the expressed human ET_A receptor-mediated Ca^{2+} mobilization was not blocked by inhibitors of gap junctions. In agreement with the binding data, the endogenous ET_{AX} receptor-mediated response was not inhibited by BQ123 even at 100 nM, whereas the expressed human ET_A receptor-mediated response was inhibited by 50% at concentrations as low as 10 nM. This further confirms that the amphibian ET_{AX} receptors are different from mammalian ET_A receptors. Finally, ET-1 enhanced the rate of progesterone-induced maturation of follicular oocytes, implying the involvement of these endogenous ET_{AX} receptors in an *in vivo* maturation process.

ET is a newly discovered 21-residue peptide that is produced by vascular endothelial cells and exhibits potent vasoconstrictor, mitogenic, and potential neuromodulatory functions. It exists in multiple isoforms (ET-1, ET-2, and ET-3) that are differentially expressed in central nervous system and peripheral tissues (for reviews, see Refs. 1 and 2). The ET family of peptides share a high degree of sequence and structural homology with the snake venom toxin S6c (3). There are two major ET receptor subtypes, denoted ET_A and ET_B (4). The ET_A receptors have high affinity for the various ETs, with a rank order of potency of ET-1/ET-2 > ET-3 ≫ S6c. In contrast, the ET_B receptor subtype displays equal affinity for all ET-related peptides, ET-1 = ET-2 = ET-3 = S6c (5, 6). Recently, Ihara *et al.* (7) described a cyclic pentapeptide (BQ123) that showed high selectivity for ET_A receptors. ETs and sarafotoxins bind to a common receptor and activate a common signal transduction pathway, principally a guanine nucleotide-binding protein-mediated activation of phospholipase C followed by an inositol triphosphate-mediated increase in intracellular Ca^{2+} levels. Expression of heterologous receptors has been demonstrated by both ligand binding and functional responses in oocytes injected with RNA derived from various cloned receptors (10–18). During our efforts to clone and characterize the ET receptors from porcine and human tissues (8, 9), we extensively used the *Xenopus laevis* oocyte system initially to test the ability of our mRNA preparations to serve as templates for the synthesis of ET receptors and then to characterize the ET receptor cDNA clones by both radioligand binding and functional (electrophysiological) response assays. In the course of those studies, we observed that the follicular membranes needed to be removed to obtain consistently reproducible results. As a result, the present study was initiated to test whether these follicular membranes displayed ET receptors. This report demonstrates for the first time the identification of novel ET receptors on follicular oocytes, their characterization, and their physiological function. We propose that this receptor be called the ET_{AX} receptor.

Materials and Methods

^{125}I-ET-1 (specific activity, 2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Unlabeled ET-1, ET-3, and S6c were from American Peptides (Santa Clara, CA). BQ123 [cyclo(D-Trp,D-Asp,L-Pro,D-Val,L-Leu)], an ET_A-selective cyclic pentapeptide antagonist, was synthesized in the Department of Peptidomimetic Research,

ABBREVIATIONS: ET, endothelin; S6c, sarafotoxin 6c; PBS, phosphate-buffered saline.

SmithKline Beecham Pharmaceuticals (King of Prussia, PA). All other chemicals were of the highest grade available.

^{125}-ET-1 binding. ^{125}I-ET-1 binding to follicular oocytes was performed in triplicate for 120 min at 18°, using 5–10 follicular oocytes/tube in buffer containing PBS, in a total volume of 100 μl. The reactions were stopped with cold PBS and filtered through Whatman GF/C filters (presoaked in 0.1% bovine serum albumin), using a Brandel cell harvester, to separate the bound ligand from free ligand. The filters were washed with 5 × 4 ml of cold PBS and counted in a γ counter at an efficiency of 75%. Saturation binding experiments were performed using increasing concentrations of ^{125}I-ET-1 (20–700 pM) in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled ET-1 and were processed as explained above. Competition binding experiments were performed using 0.3–0.4 nM ^{125}I-ET-1 in the absence and presence of increasing concentrations of the indicated compounds.

***In vitro* transcription, microinjection into *X. laevis* oocytes, and electrophysiology.** RNA transcripts were synthesized (19) from linearized human ET_A and ET_B receptor cDNA clones with T7 RNA polymerase (Stratagene) and were digested with DNase I (1 unit/μg of DNA) to remove template DNA. For microinjection, ovaries were surgically removed from *X. laevis* females (NASCO), follicle cells were dispersed, and individual oocytes were released by incubation with 2 mg/ml collagenase (Worthington) in modified Barth's medium, as described (20). After collagenase treatment and washing, the oocytes were allowed to recover overnight at 18° in Barth's medium. Stage V-VI oocytes were selected and the follicular membranes were manually removed. For each experimental group, 30–40 defolliculated oocytes were injected (Drummond injection apparatus) with 50 nl of solution containing 5 ng of *in vitro* transcribed RNA derived from human ET_A and ET_B cDNA clones and were maintained in modified Barth's medium at 18° until electrophysiological measurements were made. Electrophysiology was performed using the voltage-clamp technique, with an oocyte voltage-clamp apparatus (Warner Instruments). Oocytes were clamped at −60 mV, and the Ca^{2+}-activated Cl^- channel activity was recorded in Barth's medium at room temperature (8). In experiments where follicular oocytes were used, oocytes containing intact follicular membranes were carefully chosen. To uncouple gap junctions, both follicular oocytes and human ET_A receptor RNA-injected defolliculated oocytes (which served as a control) were treated for 20 min with either 1 mM octanol or 3 mM heptanol or for 1 min with Barth's medium gassed with 100% CO_2 before elctrophysiological measurements were made. To determine the effect of BQ123 on the ET-1-mediated electrophysiological response, both follicular oocytes and defolliculated oocytes injected with human ET_A receptor RNA were treated with 10 and 100 nM BQ123 for 5 min before electrophysiological recordings were made.

Oocyte maturation experiments. To determine the effect of ET-1 on progesterone-induced maturation, stage VI oocytes were isolated from the ovaries either manually or by 30-min treatment with collagenase. The oocytes were treated with ET-1 (0.1 μM) alone, with progesterone (3 μM) alone, or with 0.1 μM ET-1 for 30 min before the addition of progesterone.

The criteria used to score maturation were 1) the appearance of the white spot in the animal pole and 2) breakdown of the nucleus, determined by cytological examination (21). Maturation is expressed as the percentage of maximum geminal vesicle breakdown as a function of time.

Results and Discussion

Incubation of follicular oocytes with ^{125}I-ET-1 in the absence and presence of unlabeled ET-1 resulted in specific binding of ^{125}I-ET-1 to these oocytes, as shown in Fig. 1. To determine the location of these binding sites, radioligand binding studies were performed using follicular oocytes, defolliculated oocytes, and isolated follicles. Whereas follicular oocytes and follicles displayed specific ^{125}I-ET-1 binding, defolliculated oocytes did not show any binding (Fig. 1). The loss of binding in defolliculated oocytes was not due to damage to oocytes, because they were capable of expressing ET-1 binding sites when they were injected with *in vitro* transcribed ET_A receptor RNA (Fig. 1). These data show that in follicular oocytes the endogenous ET receptors are located within the follicular layer surrounding the oocytes, rather than on the oocyte plasma membrane itself. The endogenous ET-1 binding sites were maximal on freshly isolated oocytes and decreased progressively with time, reaching 10% of the original number by day 5. These endogenous ^{125}I-ET-1 binding sites were specific, saturable, and of high affinity, as shown in Fig. 2A. The nonspecific binding was between 5 and 25% of the total binding (Fig. 2A). Scatchard analysis of the specific binding from saturation binding experiments (Fig. 2A) revealed a single class of high affinity binding sites with an apparent dissociation constant (K_d) and maximum binding (B_{max}) of 50 pM and 3 fmol/10 oocytes, respectively (Fig. 2B). Competition binding experiments using ^{125}I-ET-1 and unlabeled ET-1, ET-3, S6c (ET_B-selective agonist), and BQ123 (ET_A-selective antagonist) were performed in follicular oocytes to identify the subtype of these endogenous ET binding sites. As shown in Fig. 3A, ET-1 was the most potent in displacing ^{125}I-ET-1 binding, with an IC_{50} of 0.2 nM, followed by ET-3, which was about 1000 times less potent than ET-1 (IC_{50} = 200 nM); S6c at up to 1 μM was inactive in displacing ^{125}I-ET-1 (Fig. 3A). These data compared well with those obtained in defolliculated oocytes injected with human ET_A receptor RNA, which displayed an IC_{50} of 0.8 nM, 600 nM, and >1 μM for ET-1, ET-3, and S6c, respectively (Fig. 3B). Whereas ET-1, ET-3, and S6c displayed similar binding profiles for endogenous and injected ET_A receptors, BQ123 (ET_A-selective antagonist) competed for ^{125}I-ET-1 binding to injected ET_A receptors with an IC_{50} of 20 nM (Fig. 3B), whereas it inhibited the endogenous receptors only 25% even at 1 μM (Fig. 3A). As expected, ^{125}I-ET-1 binding to defolliculated oocytes injected with human ET_B receptor RNA was displaced by unlabeled ET-1, ET-3, and S6c with similar potencies; these compounds displayed IC_{50} values of 1.0, 1.0, and 16 nM, respectively (Fig. 3C). BQ123 at up to 1 μM did not displace ^{125}I-ET-1 binding (Fig. 3C). These data clearly indicated that the ET receptors present in follicular oocytes were different from mammalian ET_B receptors and appeared to be a variant of ET_A receptors (ET_{AX}).

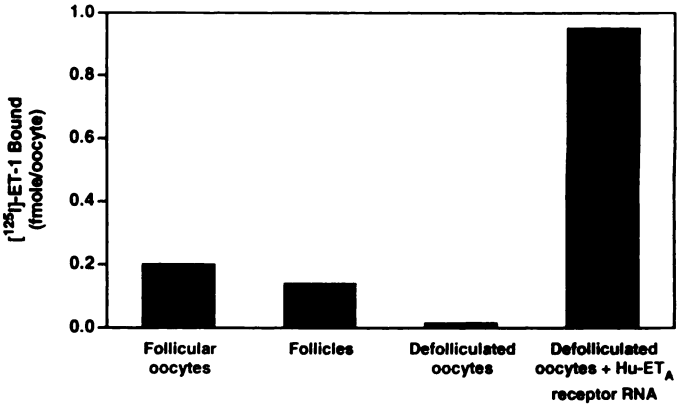

Fig. 1. Specific binding of ^{125}I-ET-1 to follicular oocytes, the removed follicular layer, defolliculated oocytes, and defolliculated oocytes injected with human ET_A receptor RNA. Each *bar* represents the mean of five separate experiments, and within each experiment 10 oocytes were used per data point.

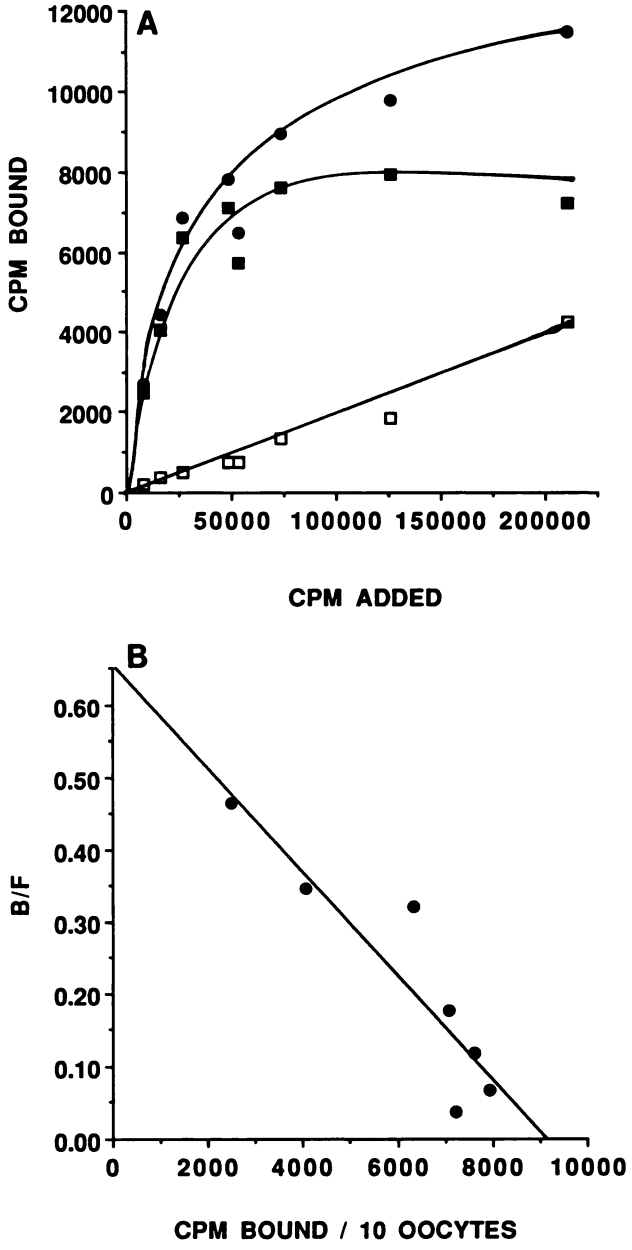

Fig. 2. A, Saturation binding of ^{125}I-ET-1 to follicular oocytes. The data presented are the mean of triplicate determinations (30 oocytes) in one of two similar experiments. ●, Total binding; □, nonspecific binding; ■, specific binding. B, Scatchard transformation of specific binding from the saturation binding experiment shown in A.

To determine whether these ET_{AX} receptors present on the follicular oocytes could trigger a functional response, we measured the ET-1-mediated electrophysiological responses in follicular oocytes (8). The oocytes that contained the surrounding follicular layer elicited a profound and rapid increase in Ca^{2+}-activated Cl^- currents upon addition of ET-1 (Fig. 4A). Defolliculation completely abolished this functional response. In contrast, defolliculated oocytes injected with ET_A receptor RNA transcripts retained their responsiveness to ET-1 (Fig. 4A). In amphibian as well as mammalian systems, gap junctions between follicle cell microvilli and oocytes provide a structural pathway for cell-cell communication by exchange of ions and small molecules between the cells (22, 23). Follicle cells have been implicated as playing a major role in amphibian oocyte

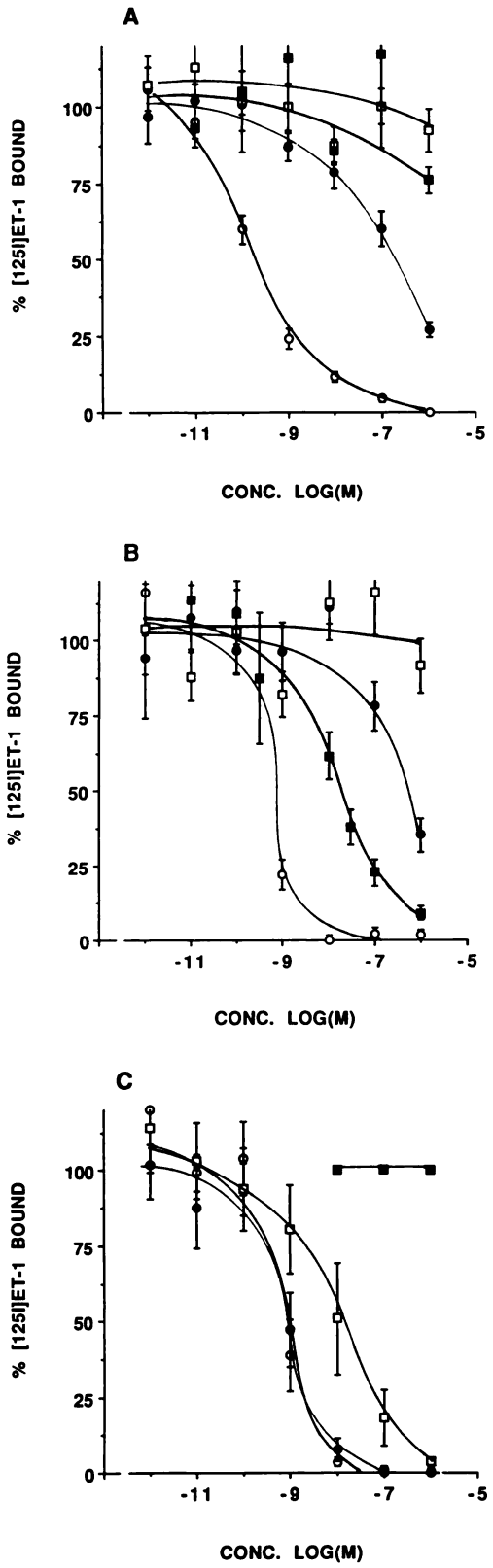

Fig. 3. Competition of ^{125}I-ET-1 binding to follicular oocytes (A), defolliculated oocytes injected with human ET_A receptor RNA (B), and defolliculated oocytes injected with human ET_B receptor RNA (C), by unlabeled ET-1 (○), ET-3 (●), BQ123 (■), and S6c (□). Data obtained in the absence of competitor were normalized (by subtracting nonspecific binding) to 100% and those obtained in the presence of 1 μM ET-1 (nonspecific binding) were considered 0%. Values are expressed as the percentage of ^{125}I-ET-1 binding. The data presented are the mean ± standard error of three or four experiments.

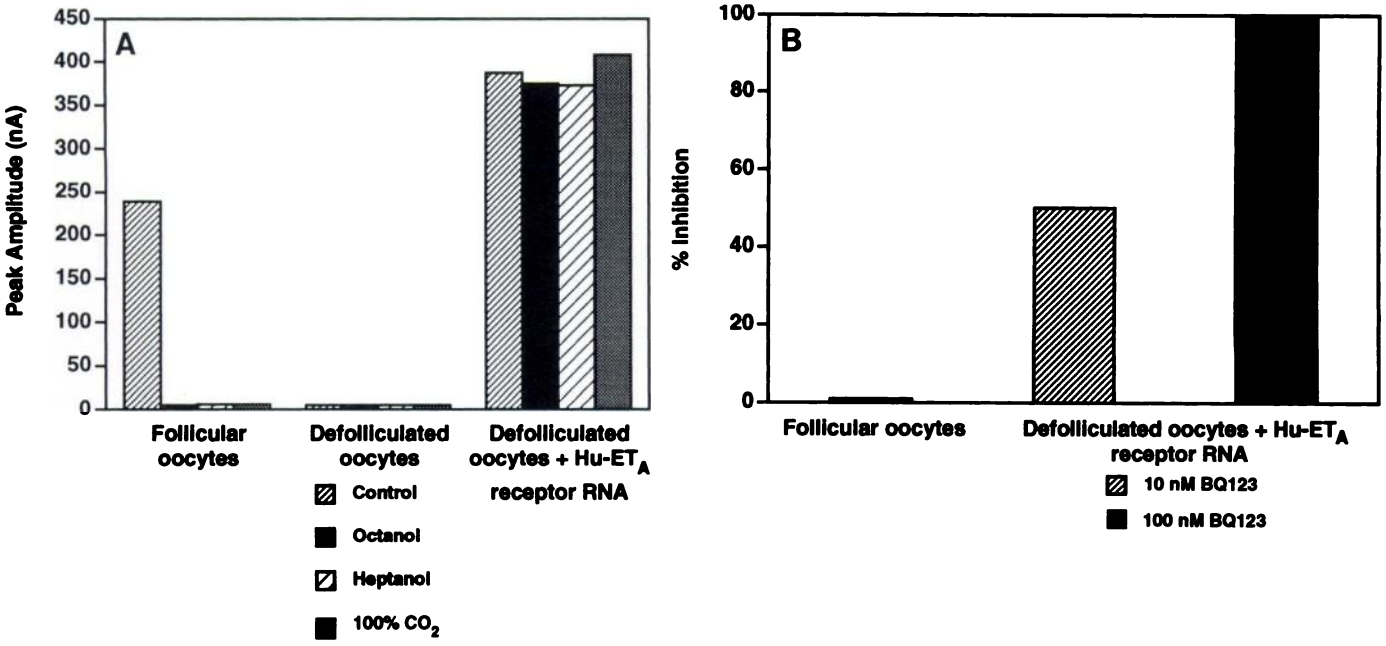

Fig. 4. A, Magnitude of the electrophysiological responses mediated by ET-1 in follicular oocytes, defolliculated oocytes, and defolliculated oocytes injected with ET_A receptor RNA. Responses are expressed as the mean of data from 10–15 oocytes. B, Effect of BQ123 on ET-1-mediated electrophysiological responses in intact oocytes and defolliculated oocytes injected with human ET_A receptor RNA.

growth and development. They are involved in steroidogenesis, amino acid uptake, protein synthesis, and several other important functions (22, 23). We therefore examined the possibility that the intrinsic ET receptors located on the oocyte follicle cells caused increases in cytoplasmic Ca^{2+} concentration by promoting the transfer of second messengers through gap junctions. ET-1-mediated electrophysiological responses were measured in oocytes that had been pretreated with octanol (24), heptanol (25), or 100% CO_2-gassed Barth's medium (26), treatments that are known to uncouple gap junctions between somatic and geminal cells. All three treatments completely abolished ET-1-mediated electrophysiological responses in follicular oocytes (Fig. 4A), and this inhibition was reversible to different extents (data not shown). However, these pretreatments did not have any effect on Ca^{2+} mobilization mediated by exogenously expressed human ET_A receptors on the defolliculated oocytes (Fig. 4A). These data suggest that octanol/heptanol treatment and cytoplasmic acidification did not perturb signal transduction by exogenously expressed ET receptors present on the plasma membrane of the oocyte but blocked the endogenous ET_{AX} receptor-mediated responses by uncoupling the communication between the follicle cells and the oocyte. Similar observations have been made with angiotensin II receptors and muscarinic receptors present on oocyte follicular membranes (27, 28). When BQ123 was tested for its effect on ET-1-mediated responses, it was observed that in follicular oocytes it did not inhibit ET-1-mediated responses at up to 100 nM, whereas in human ET_A receptor-injected oocytes it inhibited ET-1-mediated responses by 50% at 10 nM (Fig. 4B). These data agree well with the competition binding data and further confirm that the ET receptors present in follicular membranes are different from mammalian ET receptors.

The maturation of amphibian oocytes *in vivo* is regulated by hormonal factors that cause the release of progesterone by follicular cells (29). The maturation process can also be induced

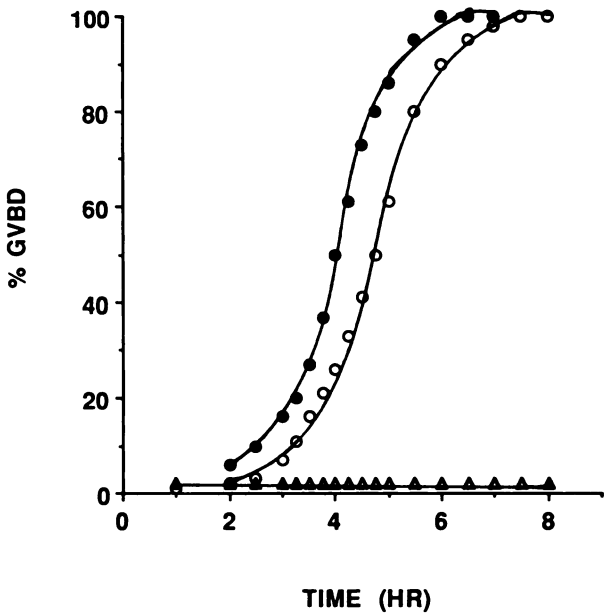

Fig. 5. Effect of ET-1 on progesterone-induced maturation. △, ET-1 (0.1 μM) alone; ○, progesterone (3 μM) alone; ●, ET-1 (0.1 μM) plus progesterone. A representative example of 15 different experiments is shown. *GVBD*, maximum geminal vesicle breakdown.

in vitro by progesterone. Progesterone-induced maturation of oocytes is significantly accelerated by concomitant exposure to acetylcholine or angiotensin II, by activation of endogenous muscarinic or angiotensin II receptors in the oocyte (27, 28). Because cellular responses to muscarinic and angiotensin II agonists include phosphoinosotide hydrolysis and Ca^{2+} mobilization, we investigated the possibility that ET-1 might influence progesterone-induced maturation by a similar mechanism.

In follicular oocytes incubated with progesterone (3 μM), ET-1 accelerated the maturation process and reduced the time in which 50% of the oocytes reached germinal vesicle breakdown (50% of maximum geminal vesicle breakdown) by 35 ± 10 min (n = 50) (Fig. 5). ET-1 alone did not induce oocyte maturation. The *in vitro* effect of ET-1 on oocyte maturation might suggest a possible physiological role for the follicular membrane ET receptors in an *in vivo* maturation process. This is the first report that describes the presence of a novel subtype of ET receptors (ET_{AX}) in follicular oocytes and their role in oocyte maturation. Molecular cloning and characterization of these novel ET_{AX} receptors and their mammalian counterparts, if any, will enable us to identify the differences between these receptors and mammalian ET_A receptors and to investigate their involvement in the regulation of mammalian ovarian function.

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Send reprint requests to: Chandrika S. Kumar, SmithKline Beecham Pharmaceuticals, Department of Molecular Genetics, UE 0548, P.O. Box 1539, King of Prussia, PA 19406–0939.