Acceleration of trophoblast differentiation by heparin-binding EGF-like growth factor is dependent on the stage-specific activation of calcium influx by ErbB receptors in developing mouse blastocysts

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SUMMARY

Heparin-binding EGF-like growth factor (HB-EGF) is expressed in the mouse endometrial epithelium during implantation exclusively at sites apposed to embryos and accelerates the development of cultured blastocysts, suggesting that it may regulate peri-implantation development in utero. We have examined the influence of HB-EGF on mouse trophoblast differentiation in vitro and the associated intracellular signaling pathways. HB-EGF both induced intracellular Ca^{2+} signaling and accelerated trophoblast development to an adhesion-competent stage, but only late on gestation day 4 after ErbB4, a receptor for HB-EGF, translocated from the cytoplasm to the apical surface of trophoblast cells. The acceleration of blastocyst differentiation by HB-EGF was attenuated after inhibition of protein tyrosine kinase activity or removal of surface heparan sulfate, as expected. Chelation of intracellular Ca^{2+} blocked the ability of HB-EGF to accelerate development, as did inhibitors of protein kinase C or

calmodulin. The absence of any effect by a phospholipase C inhibitor and the requirement for extracellular Ca^{2+} suggested that the accrued free cytoplasmic Ca^{2+} did not originate from inositol phosphate-sensitive intracellular stores, but through Ca^{2+} influx. Indeed, N-type Ca^{2+} channel blockers specifically inhibited the ability of HB-EGF to both induce Ca^{2+} signaling and accelerate trophoblast development. We conclude that HB-EGF accelerates the differentiation of trophoblast cells to an adhesion-competent stage by inducing Ca^{2+} influx, which activates calmodulin and protein kinase C. An upstream role for ErbB4 in this pathway is implicated by the timing of its translocation to the trophoblast surface.

INTRODUCTION

Blastocyst implantation in mammals depends on the interaction of differentiated trophoblast cells with endometrial tissue, the maternal-embryonic dialogue. Prior to implantation, trophoblast cells undergo a developmental program that culminates with the acquisition of adhesion competence at their apical surfaces. During blastocyst formation, newly formed outer blastomeres emerge as a polarized epithelium, the trophectoderm, which surrounds the blastocyst (Collins and Fleming, 1995). As implantation approaches, the invasive trophoblast cells arise through a phenotypic transformation of the trophectoderm that is characterized by diminished apicobasal polarity and weakened lateral cell-cell junctions (Denker, 1993). Initially, the basolateral plasma membrane is replete with integrins that mediate adhesion to a basal lamina, whereas the outward-facing apical domain is largely devoid of integrins, with the exception of $\alpha_v\beta_3$ (Hierck et al., 1993; Sutherland et al., 1993). Upon reaching the adhesive stage, additional integrins traffic to the apical plasma membrane

domain (Schultz et al., 1997), embryos attain heightened fibronectin (FN)-binding activity (Schultz and Armant, 1995) and the trophoblast cells dissociate to produce an outgrowth (Armant et al., 1986), which reflects the capacity of peri-implantation blastocysts to implant in utero (Enders et al., 1981).

The FN-binding activity of intact mouse blastocysts is used to follow integrin-mediated adhesion on the apical surface of trophoblast cells (Schultz and Armant, 1995). In accordance with trophoblast outgrowth, FN-binding activity becomes maximal on gestation day (GD) 7 (Schultz et al., 1997). It is confined to the abembryonic pole of the mouse blastocyst (Schultz and Armant, 1995; Schultz et al., 1997), which is first to become adhesive in utero and orients the embryo within the implantation chamber (Kirby et al., 1967). The onset of FN-binding activity in cultured mouse embryos coincides with the first appearance of the integrin $\alpha_5\beta_1$ on the apical surface of trophoblast cells (Schultz et al., 1997). Synthesis of $\alpha_5\beta_1$ occurs long before the blastocyst forms (Sutherland et al., 1993) and protein synthesis is not required during the 48 hour

period preceding the expression of FN-binding activity, which suggests that trafficking of this integrin to the apical plasma membrane domain is rate limiting (Schultz et al., 1997). This view is supported by the ability of brefeldin A, an inhibitor of intracellular trafficking (Klausner et al., 1992) to delay the onset of FN-binding activity (Schultz et al., 1997). In addition to the endogenous developmental program that controls blastocyst differentiation, contact with the ECM directly influences FN-binding activity. Blastocysts cultured to GD7 in the absence of an adhesive substratum exhibit a 4-fold increase in FN-binding activity after exposure to immobilized or soluble FN (Schultz and Armant, 1995).

Through recent advances, the influence of the maternal environment on blastocyst development in utero is now coming to light. During implantation, growth factors and hormones accumulate in the uterus, and are thought to regulate embryonic development through paracrine and juxtacrine interactions with the blastocyst (Stewart and Cullinan, 1997). This hypothesis is supported by demonstrations that supplementation with growth factors may advance blastocyst development in vitro. For example, epidermal growth factor (EGF), platelet-derived growth factor, transforming growth factor (TGF)-α, basic fibroblast growth factor and colony-stimulating factor-1 enhance trophoblast adhesion and migration on fibronectin (Haimovici and Anderson, 1993). Insulin-like growth factor-2, insulin and EGF stimulate human cytotrophoblast migration (Bass et al., 1994; Irving and Lala, 1995). Stage-specific products of the peri-implantation uterus, heparin-binding EGF-like growth factor (HB-EGF), calcitonin and anandamide, accelerate blastocyst differentiation in vitro (Das et al., 1994; Wang et al., 1998, 1999), providing evidence that the uterus creates an environment that promotes blastocyst implantation.

Roles for EGF family members in the regulation of preimplantation and peri-implantation development have been recognized (Das et al., 1997). During diapause, blastocyst expression of ErbB1, the EGF receptor, is downregulated and returns upon administration of estrogen to reactivate the dormant blastocyst (Paria et al., 1993). The differential regulation of the blastocyst EGF receptor during diapause and the expression of full-length ErbB1 mRNA in the blastocyst, but only a truncated mRNA in the uterine epithelium (Tong et al., 1996), points to the blastocyst as the principal target of EGF family growth factors. Indeed, EGF and TGF-α are able to stimulate synthesis of DNA, RNA and protein, cell proliferation, cavitation, blastocyst expansion and trophoblast outgrowth in vitro (Wood and Kaye, 1989; Paria and Dey, 1990; Dardik and Schultz, 1991; Adamson, 1993; Haimovici and Anderson, 1993; Machida et al., 1995). EGF induces blastocyst activation and implantation in a mouse delayed implantation model, suggesting that it may mediate the initiation of implantation by estrogen (Johnson and Chatterjee, 1993a,b). However, EGF is not expressed in the peri-implantation mouse uterus (Huet-Hudson et al., 1990), suggesting that other members of the EGF family may activate the EGF receptor or other ErbB isoforms to regulate peri-implantation trophoblast development.

A member of the EGF family, HB-EGF, was first identified as a mitogen produced by a macrophage-like cell line (Higashiyama et al., 1991). HB-EGF contains a heparin-binding site (Higashiyama et al., 1992; Onu et al., 1994), and

its activity is potentiated by heparan sulfate on the surface of target cells (Higashiyama et al., 1993). HB-EGF is expressed in the mouse uterus during implantation solely at sites of embryo apposition and its expression in rodents is regulated by steroids (Das et al., 1994; Wang et al., 1994; Zhang et al., 1994a,b). Mouse peri-implantation blastocysts express ErbB1 and ErbB4, two receptors that can bind to HB-EGF, suggesting that the peri-implantation blastocyst is targeted by HB-EGF during implantation (Paria et al., 1993, 1999; Das et al., 1994). In both mouse and human, HB-EGF appears to stimulate embryonic cell proliferation, blastocyst hatching from the zona pellucida and trophoblast outgrowth on laminin (Das et al., 1994; Martin et al, 1998). In humans, EGF increases the invasive activity of cultured cytotrophoblast cells derived from first trimester placentas (Bass et al., 1994). HB-EGF is expressed by uterine epithelial cells during the period of receptivity for implantation in the human endometrial cycle, and continues to be expressed by extravillous cytotrophoblast cells and uterine vascular endothelial cells during the first trimester, coincident with cytotrophoblast invasion of the decidua and spiral arteries (Yoo et al., 1997; Leach et al., 1999a). These studies suggest that HB-EGF is an important paracrine and autocrine regulator of trophoblast activity during implantation and early placentation.

Trophoblast intracellular signaling plays an important role in the maternal-embryonic dialogue by mediating physiological responses to uterine growth factors. The two receptors capable of binding HB-EGF, ErbB1 and ErbB4, initiate intracellular signaling and the cellular response (Riese and Stern, 1998). Ligand binding to ErbB1 stimulates receptor dimerization and tyrosine autophosphorylation at several sites. EGF activates the expression of the hCG gene through CREB phosphorylation (Matsumoto et al., 1998). This effect is dependent on protein kinase C (PKC), a Ca^{2+}-dependent protein kinase, suggesting that Ca^{2+} signaling may also be induced by EGF. In mouse preimplantation and peri-implantation development, intracellular Ca^{2+} signaling appears to play central regulatory role. Induction of intracellular Ca^{2+} signaling by either pharmacological agent or calcitonin accelerates the rate of blastocyst development in vitro (Stachecki et al., 1994a; Wang et al., 1998). Protein tyrosine phosphorylation by ErbB1 has been associated with elevated levels of intracellular Ca^{2+} (Tanaka et al., 1992), most likely through the tyrosine phosphorylation and activation of phospholipase C (PLC)-γ (Margolis et al., 1989; Rink and Merritt, 1990; Ullrich and Schlessinger, 1990), and the ensuing production of inositol 1,4,5-trisphosphate (IP_3) (Ullrich and Schlessinger, 1990; Berridge, 1993). PLC activity is required during blastocyst formation (Stachecki and Armant, 1996b), as are Ca^{2+} signaling and the Ca^{2+}-activated protein, calmodulin (Stachecki and Armant, 1996a). HB-EGF may, therefore, activate a biochemical cascade that includes the mobilization of intracellular Ca^{2+} stores and results in accelerated blastocyst differentiation.

The present study was undertaken to determine whether the ability of the in utero maternal environment to foster rapid blastocyst differentiation could be restored during in vitro culture by supplementation with HB-EGF. These treatments were carried out between GD4 and GD5, corresponding to the in utero period when trophoblast cells would be in contact with uterine epithelial cells and HB-EGF signaling takes place. In
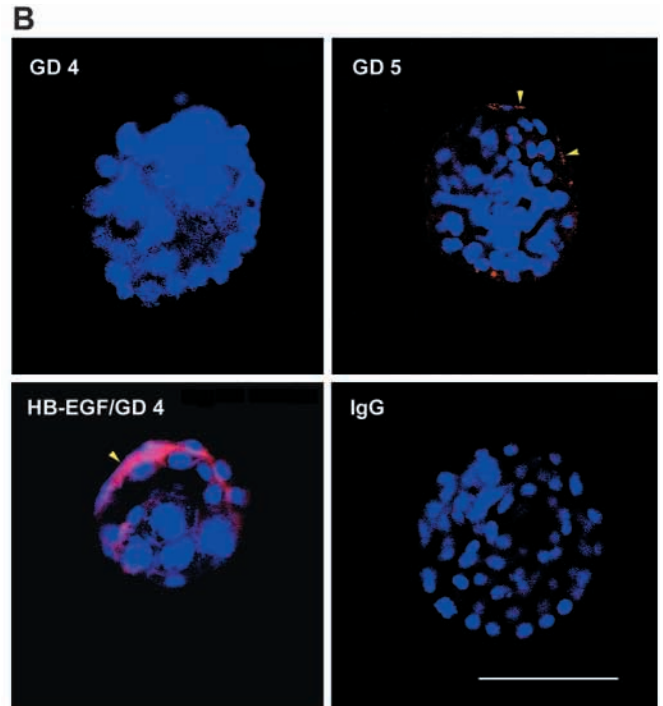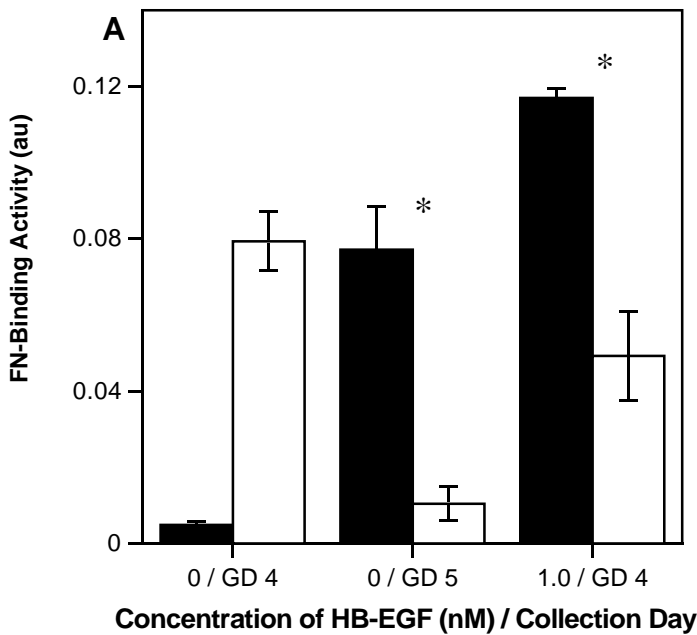
Fig. 1. Blastocyst differentiation in vitro to the adhesion-competent stage. Blastocysts were collected on GD4 or GD5, and some blastocysts collected on GD4 were exposed for 24 hours to 1 nM HB-EGF, as indicated. The rate of blastocyst differentiation was evaluated by assaying FN-binding activity (A) or expression of α_5 integrin on the apical surface of the trophoblast cells (B). (A) FN-binding activity was assayed on GD6 (solid bars) or GD7 (open bars) and is expressed in arbitrary units (au) as the mean fluorescence intensity $\pm$ s.e.m. *$P<0.05$, compared to 0/GD4 by factorial ANOVA. (B) Blastocysts collected and treated according to the above procedure were fixed without detergent permeabilization on GD6 and stained using non-immune rat IgG (IgG) or a monoclonal antibody recognizing the α_5 integrin subunit (all other panels). Indirect immunofluorescent staining of α_5 (red) and DAPI-stained nuclei (blue) was visualized by scanning laser confocal microscopy, with representative 1 μm optical sections shown here. Antibody staining of α_5 integrin subunit (arrowheads) was only observed on the apical surface of blastocysts collected on GD5 or treated on GD4 with HB-EGF. Size bar, 50 μm.

addition, we have examined the role in HB-EGF-mediated signaling of ErbB isoform localization, tyrosine kinase activity and intracellular Ca^{2+} signaling pathways.

MATERIALS AND METHODS

Production and culture of mouse embryos

Mouse embryos were generated from superovulated 5- to 8-week-old female CF1 mice (Charles River Laboratories, Wilmington, MA) mated with B6SJL/J males (Jackson Laboratory, Bar Harbor, ME), as previously described (Schultz and Armant, 1995; Khidir et al., 1995) and collected on GD3 to GD5 by flushing the oviducts or uterine horns with M2 medium (Sigma Chemical Co., St Louis, MO). Embryos were cultured in Ham's F10 medium containing 4 mg/ml bovine serum albumin (BSA; Sigma), 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from Sigma) at 37°C using a 5% CO_2 incubator. In some experiments, the medium was supplemented with 1 nM recombinant human HB-EGF (R & D Systems, Minneapolis, MN), or embryos were cultured to GD5 and then pretreated for 1 hour before exposure to HB-EGF with 10 μM genestein, 10 μM diadzein, 5 μM herbimycin A, 1 μM α-cyano-(3-ethoxy-4-hydroxy-5-phenylthiomethyl)cinnamamide (ST638), 10 μM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide-HCl (W7), 10 μM N-(6-aminohexyl)-1-naphthalenesulfonamide (W5), 10 μM calphostin C, 50 nM 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (Bisindolylmaleimide I), 10 μM 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122), 10 μM 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione (U73343), 10 μM 1,2-bis(o-aminophenoxy)ethane-N,N,N', N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) or 1 mM BAPTA (all from Calbiochem, La Jolla, CA). Pretreatments were also carried out using the Ca^{2+} channel blockers 1 μM FTX-3.3, 1 μM ϖ-conotoxin GVIA, 1 μM ϖ-conotoxin MVIIC, 2 μM ϖ-conotoxin MVIIA, 1 μM ϖ-agatoxin TK, 10 nM calcicludine or 3 μM calciseptin (all from Alomone Labs, Jerusalem, Israel). To remove trophoblast cell surface heparan sulfate, blastocysts were treated on GD5 for 30 minutes at 37°C with 1 unit/ml each of heparinases I, II and III (Sigma) before exposure to HB-EGF.

Intracellular Ca^{2+}

For determination of intracellular Ca^{2+} concentration, blastocysts were incubated for 1 hour in medium containing 5 μM fluo-3 acetoxymethyl ester (fluo-3-AM, Molecular Probes, Inc., Eugene, OR) and then rinsed free of excess fluo-3-AM in fresh medium, as previously described (Wang et al., 1998). Some embryos were loaded with fluo-3-AM in medium containing inhibitors or Ca^{2+} channel blockers, as indicated in the Results section. Fluo-3-AM-loaded embryos were then individually placed in 5 μl drops of Ham's F10 on Petri dishes flooded with mineral oil and maintained at 37°C using a digitally controlled stage warmer (Brook Industries, Lake Villa, IL). After monitoring the baseline fluorescence intensity, embryos were transferred into a drop of the same medium containing 1 nM HB-EGF.

Fluorescent images were generated by exciting fluo-3 at 450 to 490 nm and detecting the light emitted at 525 nm after enhancing the signal with a GenIISys image intensifier (DAGE-MTI, Inc., Michigan City, IN). All images were reversed and video-taped every minute for play back to a computer-based image analysis system (MCID M4, Imaging Research, St. Catherines, Ontario, Canada) that was utilized

to determine the fluorescence intensity. $[Ca^{2+}]_i$ was estimated using the following formula:

$$[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F),$$

where K_d is the dissociation constant of fluo-3 for Ca^{2+} (316 nM), F is the fluorescence intensity, F_{min} is the background fluorescence, and F_{max} is the maximal fluorescence obtained by equilibrating cytoplasmic and extracellular Ca^{2+} using 5 μM ionomycin. The data shown depict single embryos recorded at 1 minute intervals that are representative of a minimum of 10 embryos for each treatment.

FN-binding activity

FN-binding activity was upregulated by exposure to 50 μg/ml FN-120 (Life Tech, Gaithersburg, MD) for 1 hour and assayed using fluorescent-green, 1.0 μm, polystyrene microspheres (Bang's Laboratories, Carmel, IN) coated with FN-120, as previously described (Schultz and Armant, 1995; Wang et al., 1998). The fluorescence intensity of the bound microspheres was quantified over the abembryonic pole of each blastocyst using computer-based image analysis (Schultz and Armant, 1995).

Confocal immunofluorescence microscopy

Blastocysts were fixed for 30 minutes at room temperature in PBS containing 3% paraformaldehyde and washed through 2 drops of 0.15 M glycine, pH 7.2. For staining of ErbB receptors, embryos were permeablized by treatment with 0.1% Triton ×100 (Sigma) for 15 minutes at room temperature. Five drops of PBS containing 10 mg/ml BSA (PBS/BSA) were used to rinse embryos after each incubation throughout this procedure. All antibodies were prepared at 10 μg/ml in PBS/BSA and incubated overnight with blastocysts at 4°C. Primary antibodies used in this study were purified rat monoclonal antibodies against the extracellular domains of mouse α_5 (5H10-27, rat IgG) and β_1 (9EG7, rat IgG) integrin subunits (PharMingen, San Diego, CA), or purified polyclonal rabbit IgG recognizing ErbB1-4 (K138, 115B, K028 and C058, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Primary antibody controls were 10 μg/ml non-immune rat IgG or rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Primary antibodies were detected using 10 μg/ml Texas Red-conjugated goat anti-rat or goat anti-rabbit IgG (Jackson) and nuclei were stained with 10 μg/ml 4′,6′,-diamidino-2-phenylindole, HCl (DAPI; Calbiochem). Embryos were incubated with a cocktail of secondary antibodies and DAPI at 37°C for 60 minutes, rinsed, mounted on slides with permanent mounting medium (Chemicon, Temecula, CA) and stored in the dark at 4°C. Antigens labeled with Texas-Red (red) and nuclei stained with DAPI (blue) were viewed in a Zeiss (Thornwood, NY) 310 confocal scanning laser microscope using excitation wavelengths of 543 nm and 364 nm, respectively. Images shown in the Results were representative of at least 15 embryos that produced similar staining patterns.

Statistical analysis

All experiments were repeated at least three times. Values reported for FN-binding activity represent the mean ± s.e.m. of at least 20 blastocysts pooled from all replicate experiments. Differences in the FN-binding activity on GD6 between treatment groups were tested for significance using a one-way ANOVA and the Bonferroni/Dunn post-hoc test. For simultaneous comparison of FN-binding activity on GD6 and GD7, a factorial ANOVA was used (Fig. 1 only).

RESULTS

Exposure of blastocysts to HB-EGF in vitro promotes differentiation comparable to embryos developing in utero

To determine whether factors present in the uterus between

GD4 and GD5 influence trophoblast cell differentiation, blastocysts were harvested on either GD4 (zona intact) or GD5 (hatched) and cultured in vitro to the adhesion-competent stage. Blastocysts were assayed for FN-binding activity on GD6 and GD7 to assess the rate of development. Embryos that were retained in utero until GD5 developed significantly faster than embryos collected on GD4, as characterized by a shift in the maximal FN-binding activity from GD7 to GD6 (Fig. 1A; $P<0.05$). Since HB-EGF is expressed in the uterus at the onset of implantation, we assessed the development of blastocysts collected on GD4 in culture medium supplemented with HB-EGF. Addition of 1 nM HB-EGF to blastocysts collected on GD4 accelerated trophoblast differentiation to the same extent as in utero development to GD5 (Fig. 1A).

Previous investigations have demonstrated a temporal correlation between maximal FN-binding activity and expression of the integrin $\alpha_5\beta_1$ on the apical surface of trophoblast cells during normal and calcitonin-stimulated preimplantation development (Schultz et al., 1997; Wang et al., 1998). Therefore, $\alpha_5\beta_1$ was assessed by immunofluorescence confocal microscopy on the surface of non-permeablized blastocysts on GD6. In agreement with the expression of maximal FN-binding activity, α_5 (Fig. 1B) and β_1 (data not shown) appeared on the apical surface of embryos stimulated to develop faster by either treatment with HB-EGF or in utero development to GD5. No $\alpha_5\beta_1$ staining was found on the surface of non-treated blastocysts collected on GD4. These findings suggest that exposure of blastocysts to HB-EGF, either in utero or by its addition to culture medium, accelerates subsequent development in vitro to an adhesion-competent stage.

The responsiveness of blastocysts to HB-EGF is temporally regulated

Acceleration of blastocyst differentiation by treatment on GD4 with calcitonin or anandamide requires only 1 hour of exposure (Wang et al., 1998, 1999). A 1 hour HB-EGF treatment on GD4 failed to stimulate blastocyst differentiation (data not shown); however, maximal FN-binding activity shifted to GD6 after exposure to 1 nM HB-EGF at 0900 hours on GD5 (Fig. 2). To determine the onset of blastocyst responsiveness to HB-EGF, blastocysts were exposed to 1 nM HB-EGF during specific periods on GD4 and FN-binding activity was estimated on GD6 to determine whether development was accelerated. HB-EGF accelerated development only when embryos were treated at 2000 hours on GD4 or later, and a 1 hour treatment was as effective as 24 hours of exposure (Fig. 2).

Trafficking of ErbB4 correlates with blastocyst responsiveness to HB-EGF

Induction of intracellular signaling by HB-EGF may be dependent on any of the four ErbB isoforms, either through direct binding to ErbB1 or ErbB4, or through receptor codimerization (Riese and Stern, 1998). We have examined the expression of ErbB isoforms by indirect immunofluorescent labeling and confocal microscopy using blastocysts that were fixed and permeablized on GD4, either at 1500 hours when blastocysts did not respond to HB-EGF, or at 2000 hours when blastocysts were first responsive to HB-EGF. Blastocysts expressed all four ErbB isoforms at 1500 hours; however, the pattern of staining for ErbB1, ErbB2 and ErbB3 indicated
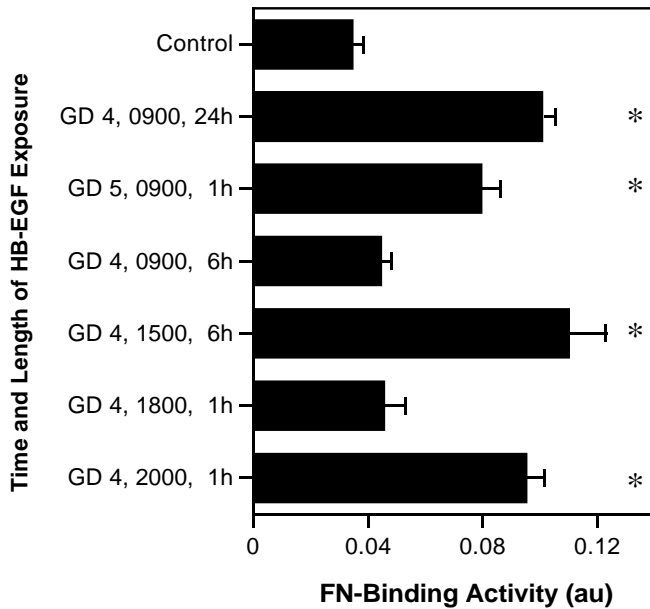
Fig. 2. Temporal regulation of the response to HB-EGF. Blastocysts collected on GD4 were exposed to 1 nM HB-EGF during the indicated time periods. The times of exposure, indicated by GD and hour of the day, and the length of exposure in hours are provided on the ordinate. Untreated embryos served as control (Control). The rate of blastocyst differentiation was determined by assaying FN-binding activity on GD6, as in Fig. 1A. *$P<0.05$, compared to control.

surface localization, while ErbB4 appeared to be localized within the cytoplasm (Fig. 3). At 2000 hours, ErbB4 was localized on the surface of the blastocyst, demonstrating that translocation of ErbB4 to the apical surface of trophoblast cells occurred close to the time that blastocysts became responsive to HB-EGF.

Blastocyst responsiveness to HB-EGF is associated with intracellular Ca^{2+} signaling

It has been reported that EGF-dependent protein tyrosine phosphorylation elevates cytosolic free Ca^{2+} either through mobilization of intracellular stores or influx through Ca^{2+} channels (Moolenar et al., 1986; Peppelenbosch et al., 1991; Cohen et al., 1996). Preimplantation development is accelerated by calcitonin or pharmacological agents that raise intracellular Ca^{2+} levels (Stachecki et al., 1994a,b; Wang et al., 1998). To determine whether HB-EGF regulates preimplantation development by elevating intracellular Ca^{2+}, we have measured intracellular Ca^{2+} levels in cultured blastocysts on GD5 after administration of 1 nM HB-EGF. Indeed, intracellular Ca^{2+} increased within 1 minute of HB-EGF addition and remained elevated for approximately 10 to 30 minutes (Fig. 4). All cells within the embryo appeared to respond in this manner (Fig. 4A). However, HB-EGF induced intracellular Ca^{2+} signaling only after 2000 hours on GD4 (Fig. 4B), coincident with ErbB4 trafficking and the onset of blastocyst responsiveness to HB-EGF. No change in intracellular Ca^{2+} level was observed in vehicle-treated blastocysts. After HB-EGF treatment, Ca^{2+} levels exceeded 350 nM, which effectively accelerates preimplantation development (Stachecki and Armant, 1996a).

Table 1. Signaling pathways essential for HB-EGF activity

Inhibitors	Target	FN-binding activity (au)
Vehicle‡		0.0382±0.0043
No inhibitor		0.0979±0.0060*
10 μM BAPTA-AM	Intracellular Ca^{2+}	0.0444±0.0056
10 μM W7	Calmodulin	0.0452±0.0064
10 μM W5	Less-active analogue of W7	0.0937±0.0100*
10 μM Calphostin C	PKC	0.0449±0.0063
50 nM Bisindolylmaleimide I	PKC	0.0320±0.0049

Embryos were treated for 1 hour on GD5 with the indicated inhibitors. They were then incubated for 1 hour with 1 nM HB-EGF. W7 and W5 were also included during exposure to HB-EGF. Subsequent embryo culture was conducted in medium without HB-EGF or inhibitor. FN-binding activity, shown here as mean ± s.e.m., was measured on GD6 to assess the rate of blastocyst differentiation.
  ‡No exposure to HB-EGF or inhibitor.
 *$P<0.05$, compared to Vehicle.

Blastocyst responsiveness to HB-EGF depends on protein tyrosine kinase activity, heparan sulfate and intracellular Ca^{2+} signaling

We have investigated the role of tyrosine phosphorylation in HB-EGF-induced acceleration of trophoblast development, since ErbB receptors possess protein tyrosine kinase domains (Riese II and Stern, 1998). Embryos were treated for 1 hour on GD5 with 10 μM of a broad-spectrum protein tyrosine kinase inhibitor, genestein, or its less-active structural analog, diadzein. Trophoblast differentiation was estimated by measuring FN-binding activity on GD6. Genestein, but not diadzein, blocked the stimulatory effect of HB-EGF on development (Fig. 5). Two other tyrosine kinase inhibitors, herbimycin A and ST638, effectively attenuated any significant increase in FN-binding activity on GD6, verifying that tyrosine phosphorylation was required for HB-EGF-mediated acceleration of trophoblast differentiation.

HB-EGF activity is potentiated by its interaction with cell surface heparan sulfate (Higashiyama et al., 1993). After treating blastocysts on GD5 for 30 minutes with heparinase, HB-EGF failed to accelerate blastocyst differentiation (Fig. 5), demonstrating that the interaction between HB-EGF and its receptors is augmented by cell surface heparan sulfate. This finding is consistent with a recent report that HB-EGF binding to its receptor is heparan sulfate-dependent in mouse blastocysts (Paria et al., 1999).

Ca^{2+} signaling appears to be an important regulatory pathway during preimplantation development (Stachecki and Armant, 1994a,b; Wang et al., 1998), consistent with the ability of HB-EGF to elevate intracellular Ca^{2+}. To more firmly establish a role for intracellular Ca^{2+} signaling in the acceleration of blastocyst differentiation by HB-EGF, we treated embryos for 1 hour before HB-EGF exposure with 10 μM BAPTA-AM, an intracellular Ca^{2+} chelator. This level of BAPTA-AM successfully blocks Ca^{2+} signaling in embryos treated with Ca^{2+} ionophore or calcitonin (Stachecki and Armant, 1996a; Wang et al., 1998). Blastocysts treated with BAPTA-AM did not express elevated FN-binding activity on GD6 in response to HB-EGF exposure (Table 1). This experiment clearly shows that the acceleration of development by HB-EGF was dependent on its ability to induce Ca^{2+} signaling.

Fig. 3. Cellular localization of ErbB
receptors in developing blastocysts.
Blastocysts were fixed and permeabilized on
GD4 when blastocysts were either non-
responsive (1500 hours) or responsive (2000
hours) to HB-EGF. They were then stained
using non-immune rabbit IgG (F) or
polyclonal rabbit antibodies recognizing
ErbB1 (A), ErbB2 (B), ErbB3 (C) or ErbB4
(D,E). Antigens and nuclei were visualized
using Texas Red-conjugated secondary
antibody and DAPI, as in Fig. 1B. ErbB1-3
were resident on the trophoblast apical
surface at 1500 hours (A-C), while ErbB4
was present only in the cytoplasm (D).
ErbB4 translocated to the apical surface by
2000 hours (E). Size bar, 50 μm.
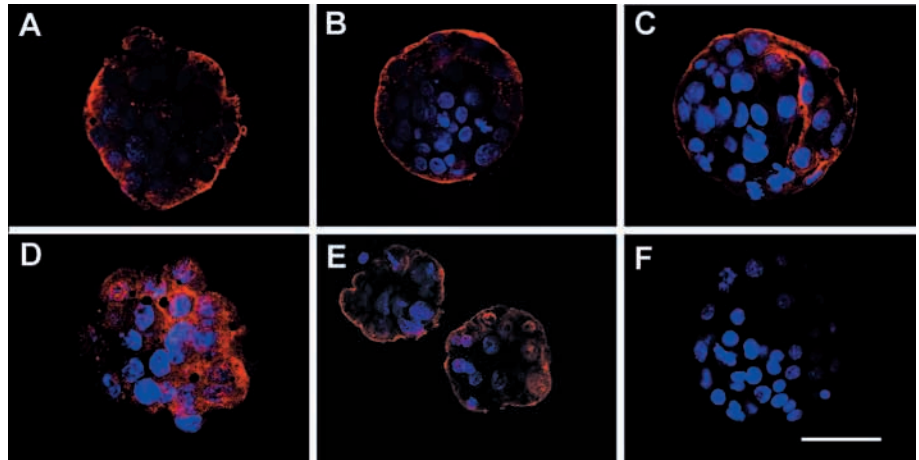
The dependence of HB-EGF activity on intracellular Ca^{2+}
signaling suggested that Ca^{2+}-dependent regulatory molecules
mediate the acceleration of trophoblast differentiation by HB-
EGF. Protein kinase C and calmodulin are Ca^{2+}-dependent
proteins that regulate cell proliferation and differentiation
(Means, 1994; Livneh and Fishman, 1997). To determine
whether these regulatory proteins were active downstream of
Ca^{2+}, we treated blastocysts with inhibitors of either protein
kinase C (calphostin C and bisindolylmaleimide I) or

calmodulin (W7) before exposure to HB-EGF on GD5. All
three inhibitors abolished the ability of HB-EGF to accelerate
trophoblast development (Table 1), suggesting that signaling
pathways activated by calmodulin and protein kinase C
regulate differentiation of the blastocyst to an adhesion-
competent stage. The less-active analog of W7, W5, produced
no attenuation of HB-EGF-induced acceleration of trophoblast
differentiation (Table 1).
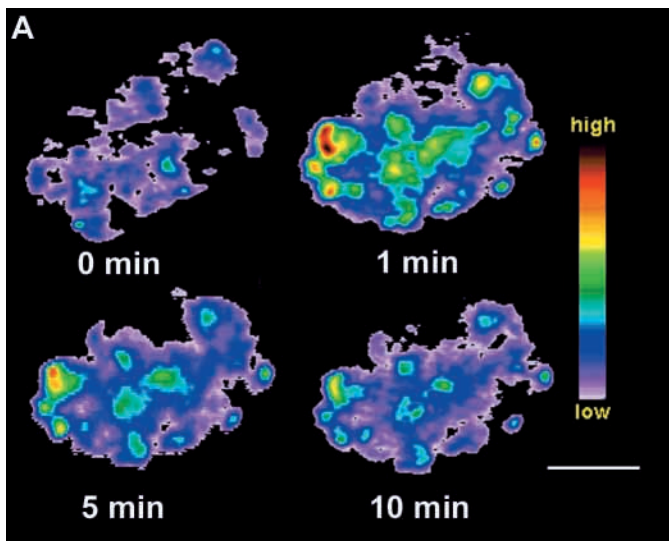
Fig. 4. Induction of intracellular Ca^{2+} signaling by HB-EGF.
Blastocysts were loaded with fluo-3-AM for 1 hour before exposure
to 1 nM HB-EGF. (A) The relative level of intracellular Ca^{2+} on GD5
in a representative blastocyst is indicated by the fluorescent intensity,
which is displayed in pseudocolor according to the color bar. At 0
minutes, the blastocyst was transferred to a drop containing 1 nM
HB-EGF. Intracellular Ca^{2+} levels increased thereafter throughout
the blastocyst and returned to baseline within 30 minutes. Size bar,
25 μm. (B) The concentration of intracellular Ca^{2+} was estimated in
blastocysts after exposure to 0 (Vehicle) or 1 nM (HB-EGF) HB-
EGF. The time of exposure during development, in GD and hour of
the day, is indicated in the upper right corner of each graph. Each
graph depicts the average concentration of intracellular Ca^{2+}
integrated over an entire representative embryo.
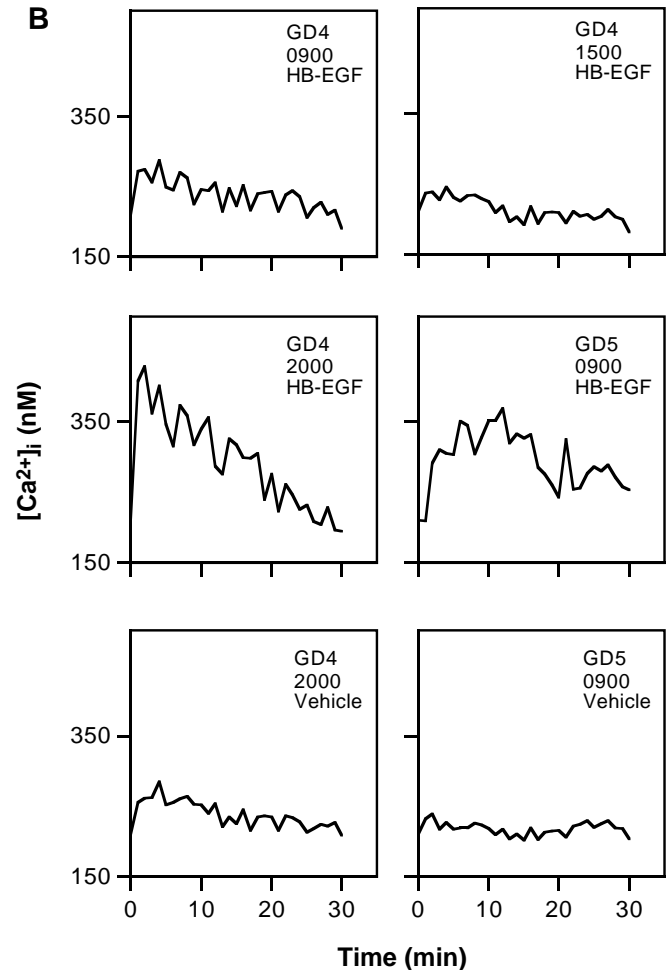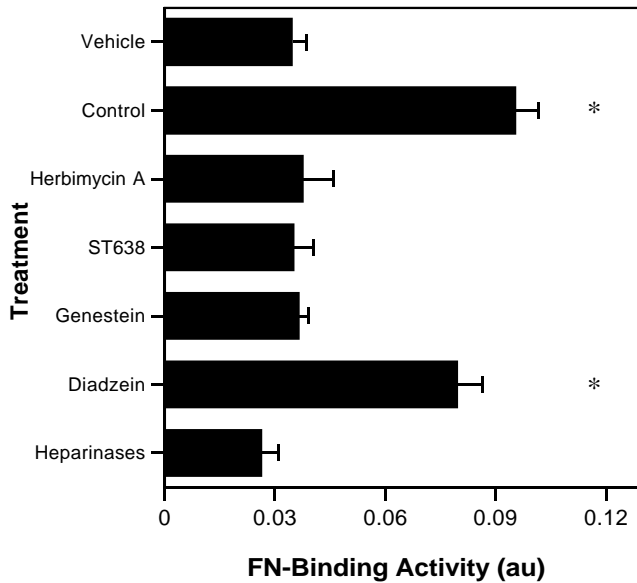
Fig. 5. Requirements for protein tyrosine kinase activity and cell surface heparan sulfate in the response of blastocysts to HB-EGF. Embryos were collected on GD4 and cultured either without further treatment (Vehicle) or with exposure to 1 nM HBEGF on GD5 for 1 hour (Control). Prior to HB-EGF addition, some embryos were treated for 1 hour with 10 µM genestein, 10 µM diadzein, 5 µM herbimycin A or 1 µM ST638. Embryos were also treated with 1 unit/ml each of heparinases I, II and III before HB-EGF exposure. The rate of preimplantation development was assessed by assaying FN-binding activity on GD6, as in Fig. 1A. *$P<0.05$, compared to Vehicle.

HB-EGF opens Ca^{2+} channels

Mouse preimplantation embryos are capable of regulating the concentration of free cytosolic Ca^{2+} through its release from IP$_3$-mediated Ca^{2+} stores upon activation of PLC (Stachecki and Armant, 1996a,b). EGF induces tyrosine phosphorylation and activation of PLC-γ (Nishibe et al., 1990). To determine if IP$_3$ production by PLC-γ mediates HB-EGF-induced Ca^{2+} mobilization and acceleration of blastocyst differentiation, we treated blastocysts on GD5 with a PLC inhibitor, U73122, before exposure to HB-EGF. Neither U73122 nor its less-active structural analogue, U73343, blocked HB-EGF-induced elevation of intracellular Ca^{2+} (Fig. 6A) or acceleration of development (Fig. 6B), indicating that PLC did not mediate the effects of HB-EGF. We have previously found that 10 µM U73122 (IC$_{50}$≈2 µM) will prevent Ca^{2+} mobilization in mouse preimplantation embryos treated with ethanol or lysophosphatidic acid (Stachecki and Armant, 1996b), providing evidence that the inhibitor is effective in blocking PLC activity.

To determine whether HB-EGF-induced intracellular Ca^{2+} signaling was dependent on Ca^{2+} influx, we examined the effects of HB-EGF on blastocysts maintained in medium supplemented with 1 mM BAPTA to chelate extracellular Ca^{2+}. In this medium, the ability of HB-EGF to significantly increase intracellular Ca^{2+} levels (Fig. 6A) and accelerate development (Fig. 6B) was attenuated, suggesting that HB-EGF induces Ca^{2+} influx. Blastocysts were then treated on GD5 with inhibitors of L-, P-/Q-, Q-, or N-type Ca^{2+} channels (Table 2)

Table 2. Effect of Ca^{2+} channel blockers on HB-EGF activity

Blockers	Target channel type	FN-binding activity (au)
Vehicle‡		0.0345±0.0041
No blocker		0.0985±0.0070*
1 µM ϖ-conotoxin GVIA	N-	0.0475±0.0074
2 µM ϖ-conotoxin MVIIA	N-	0.0225±0.0047
1 µM ϖ-conotoxin MVIIC	Q-	0.0815±0.0084*
1 µM FTX-3.3	P-/Q-	0.101±0.0097*
3 µM Calciseptine	L-	0.0820±0.0085*
Combination:		0.0945±0.0092*
1 µM ϖ-agatoxin TK	P-/Q-	
10 nM Calcicludine	L-	

Embryos were treated for 1 hour on GD5 with the indicated Ca^{2+} channel blockers. They were then incubated for 1 hour with 1 nM HB-EGF in the continuing presence of the channel blockers. Subsequent culture was conducted and FN-binding activity measured on GD6, as in Table 1.
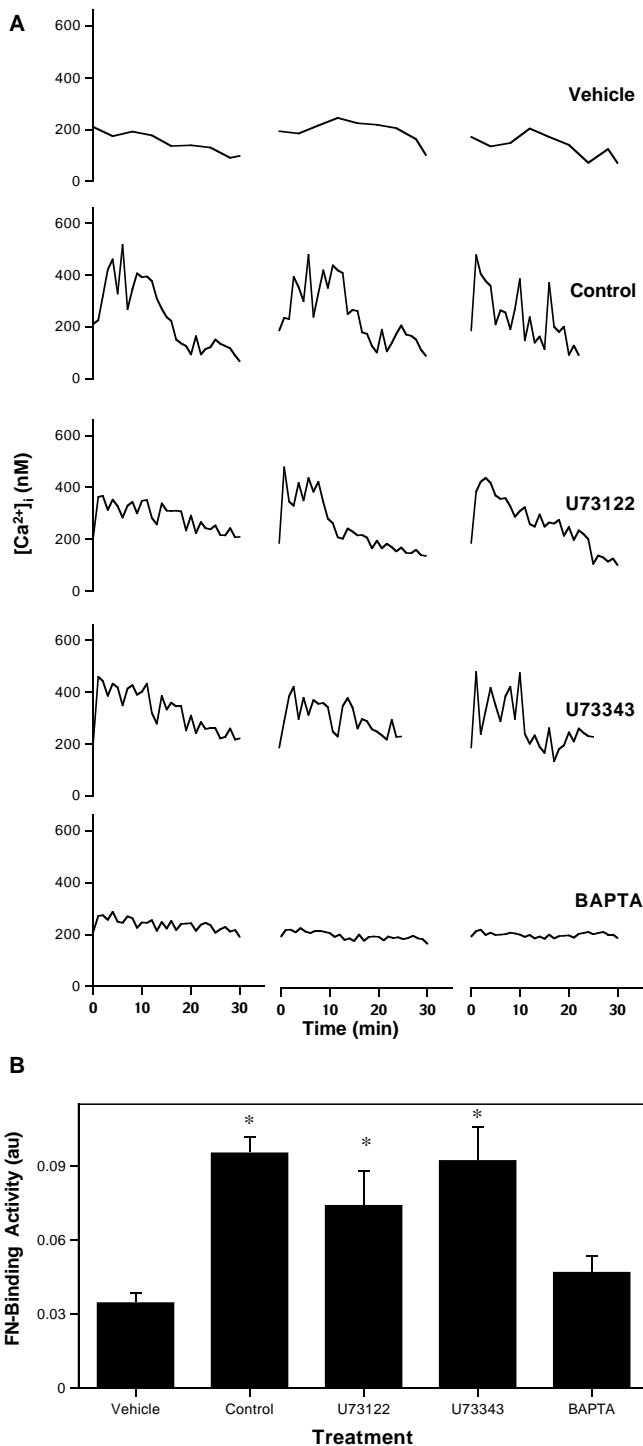
‡No exposure to HB-EGF or inhibitor.
*$P<0.05$, compared to Vehicle.

and exposed to 1 nM HB-EGF in the continued presence of the inhibitors. Among the Ca^{2+} channel blockers, only the N-type antagonist, ϖ-conotoxin GVIA, prevented HB-EGF-induced intracellular Ca^{2+} signaling (Fig. 7). This blocker, as well as another N-type channel blocker, ϖ-conotoxin MVIIA, inhibited the acceleration of trophoblast development by HB-EGF (Table 2). Blockers of other channel types, used both singly and in combination, altered neither the ability of HB-EGF to induce Ca^{2+} transients nor its impact on blastocyst differentiation. These results suggest that HB-EGF signaling activates N-type Ca^{2+} channels to initiate Ca^{2+} influx and the subsequent activation of calmodulin and protein kinase C, which accelerates blastocyst development to an adhesion-competent stage.

DISCUSSION

The absence of uterine-produced growth factors perhaps delays embryonic development in vitro. We observed a delay in trophoblast differentiation of at least 24 hours when blastocyst were collected on GD4, compared with the embryos that continued to develop in utero for an additional day. Although fewer embryos were recovered, we were successful in flushing blastocysts that had hatched and initiated contact with the uterine epithelium on GD5. In embryos collected on GD5, the expression of peak FN-binding activity and precocious α$_5$β$_1$ integrin translocation on GD6 occurred very close to the time in utero (0400, GD6) when the blastocyst first contacts and adheres to the endometrial basement membrane (Blankenship and Given, 1992). The ability of HB-EGF to enhance preimplantation development and its implantation-site-specific expression in the uterine luminal epithelium (Das et al., 1994), suggests that it may facilitate the acquisition of adhesion-competence by trophoblast cells in utero. By following FN-binding activity and surface localization of α$_5$β$_1$ integrin, we determined that HB-EGF shifted the developmental program. Supplementation with 1 nM HB-EGF accelerated blastocyst differentiation, bringing it close to the rate attained by blastocysts collected on GD5. Our results are consistent with an earlier observation that HB-EGF treatment produces

A

1998). Accordingly, tyrosine kinase activity was required for the rapid differentiation of blastocysts treated with HB-EGF. This result was verified using three different tyrosine kinase inhibitors. As previously described (Higashiyama et al., 1993), cell surface heparan sulfate was also required. However, higher concentrations of HB-EGF overcome the inhibition by heparinases (Paria et al., 1999), suggesting that heparan sulfate may enhance HB-EGF-receptor affinity in blastocysts.

The finding that ErbB4 translocation was temporally correlated with both blastocyst responsiveness to HB-EGF and the onset of Ca^{2+} signaling induced by HB-EGF is remarkable. These data support accumulating evidence that ErbB4 is the major mediator of HB-EGF binding to mouse blastocysts and other cell types (Elenius et al., 1997; Paria et al., 1999). Expression of ErbB1-3 on the apical surface of trophoblast cells was not sufficient to mediate responses to HB-EGF, suggesting that HB-EGF induced intracellular signaling specifically through ErbB4. It should be noted, however, that ligation of ErbB isoforms may induce receptor heterodimerization (Gamett et al., 1997) and that signaling induced by EGF family growth factors commonly requires synergism among ErbB isoforms (Alimandi et al., 1995; Zhang et al., 1996). Therefore, it is possible that, in addition to ErbB4, other ErbB isoforms participate in signaling initiated by HB-EGF in trophoblast cells.

Based on the inhibitory effects of BAPTA-AM, we concluded that elevation of intracellular Ca^{2+} is essential for the acceleration of blastocyst development by HB-EGF. This is consistent with previous reports that this second messenger is an important regulator of preimplantation development (Stachecki et al., 1994a,b; Wang et al., 1998). Indeed, ethanol-induced elevation of intracellular Ca^{2+} rapidly alters the expression of as many as 3.5% of blastocyst transcripts, including the increased expression of arginase and c-Myc (Rout et al., 1997; Leach et al., 1999b). Arginase produces ornithine, a precursor of polyamines, which promote cell proliferation through their ability to stabilize replicating DNA and package chromatin (Tabor and Tabor, 1984). The physiological effects of c-Myc include promotion of cell proliferation, inhibition of terminal differentiation and induction of apoptosis (Classon et al., 1987; Iguchi-Ariga et al., 1987; Eilers et al., 1991). Protein kinase C and calmodulin, two factors operating downstream to Ca^{2+} (Kishimoto et al., 1980; Means and Redman, 1980; Sasaki and Hidaka, 1982), mediate the stimulatory effects of Ca^{2+} on cellular growth (Cheung, 1980; Chafouleas et al., 1982; Means et al., 1991; Means, 1994; Livneh and Fishman, 1997). We reported that

B

enlarged blastocyst outgrowths (Das et al., 1994), perhaps due to the precocious onset of trophoblast development. It is possible that other trophic factors present in the uterus between GD4 and GD5 (e.g., calcitonin (Wang et al., 1998)) accelerate the developmental program of blastocysts harvested on GD5.

Protein tyrosine phosphorylation is the primary proximal mediator of signaling through ErbB isoforms (Riese and Stern, 1997). Ligation of ErbB receptors initiates autophosphorylation (Cohen et al., 1996), an essential step for mitogen-activated protein kinase activation (Eguchi et al.,
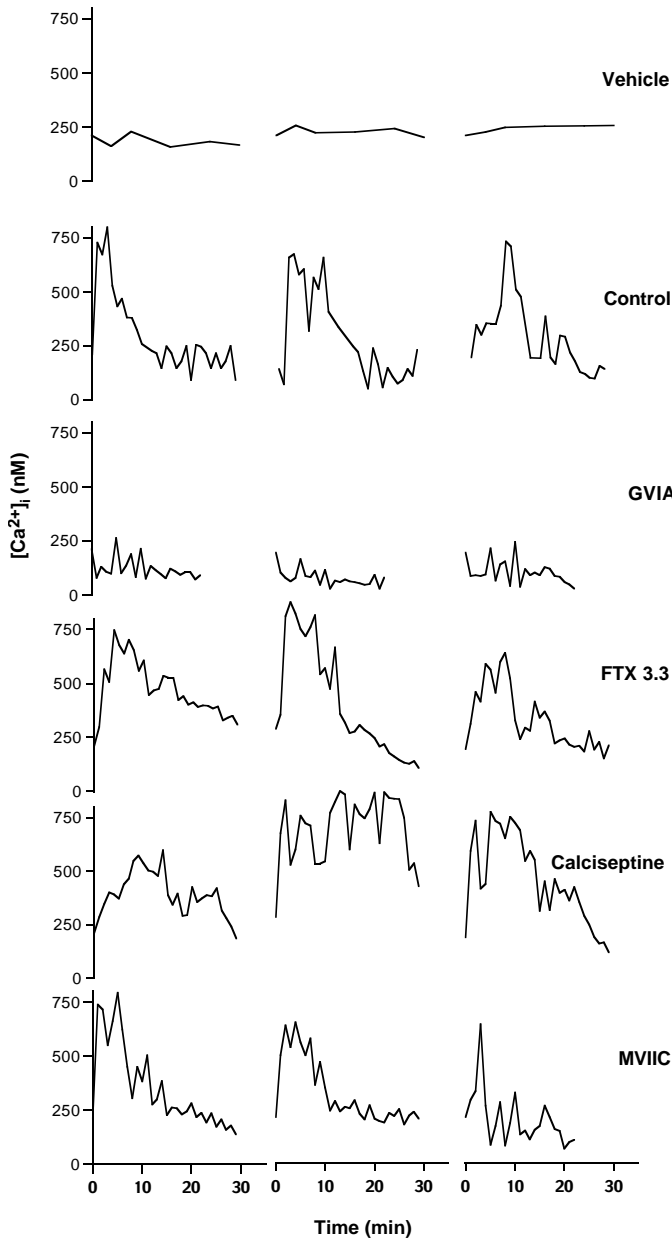
Fig. 7. Effect of Ca^{2+} channel blockers on HB-EGF-induced intracellular Ca^{2+} transients. Blastocysts were treated on GD5 for 1 hour without (Control) or with the indicated Ca^{2+} channel blockers and fluo-3-AM. They were then exposed to 1 nM HB-EGF in the continuing presence of blockers. Intracellular Ca^{2+} concentrations were estimated as in Fig. 4B. Each graph depicts a representative embryo that was exposed at 0 minutes to 0 nM (Vehicle) or 1 nM HB-EGF.

inhibition of calmodulin or protein kinase C abolished the biological activity of HB-EGF, supporting our conclusion that intracellular Ca^{2+} signaling induced by HB-EGF is responsible for the acceleration of blastocyst development.

The temporal proximity of ErbB4 surface expression and the ability of HB-EGF to elevate intracellular Ca^{2+} linked ErbB4 and Ca^{2+} as the major mediators of HB-EGF activity. The rate of preimplantation development is accelerated by ethanol-induced mobilization of Ca^{2+} from intracellular stores, due to

the ability of ethanol to activate PLC and produce IP_3 (Stachecki and Armant, 1996a,b). Activation of ErbB1 induces phosphorylation of PLC-γ (Margolis et al., 1989), suggesting that PLC is the major mediator of ErbB1-associated intracellular Ca^{2+} signaling. Ca^{2+} transients have been induced in cultured NIH 3T3 cells by EGF through a mechanism that requires ErbB1 activation and phosphorylation of PLC-γl (Cohen et al., 1996). In addition, EGF may activate PLC through a G-protein-dependent mechanism (Teitelbaum et al., 1990). Heregulin, which binds to ErbB4 and not ErbB1 (Riese and Stern, 1998), is incapable of triggering Ca^{2+} mobilization through the PLC/IP_3 pathway (Cohen et al., 1996). Since ErbB4 appear to be the major mediator of HB-EGF activity in blastocysts, we were not surprised to find that HB-EGF induced Ca^{2+} signaling independently of PLC. These findings lend new support to the view that the activity of HB-EGF is specifically mediated by ErbB4.

The ability of HB-EGF to elevate intracellular Ca^{2+} and accelerate preimplantation development was dependent on extracellular Ca^{2+}, suggesting a mechanism involving Ca^{2+} influx. L-type Ca^{2+} channels can be activated by protein tyrosine kinase, while T-type Ca^{2+} channels are activated by tyrosine phosphatase (Cataldi et al., 1996; Arnoult et al., 1997; Strauss et al., 1997), providing evidence that receptor tyrosine kinase activity of ErbB4 may regulate Ca^{2+} channel function. We report that an N-type Ca^{2+} channel blocker attenuated the ability of HB-EGF to elevate intracellular Ca^{2+}, whereas inhibition of other Ca^{2+} channel types was without effect. This exciting finding was supported by our observation that the blockers similarly affected blastocyst differentiation. This is the first report linking N-type Ca^{2+} channels with HB-EGF-induced intracellular signaling.

Yoshinaga (1994) has proposed that the induction of Ca^{2+} influx by trophoblast ErbB receptors may alter the adhesion of luminal epithelial cells during the blastocyst apposition stage of implantation. During apposition, HB-EGF can function as both a juxtacrine signaling molecule (Das et al., 1994) and a mediator of adhesion between the apical surfaces of trophoblast and epithelial cells (Raab et al., 1996). In his hypothesis, Yoshinaga (1994) predicted that juxtacrine signaling through trophoblast ErbB receptors would induce Ca^{2+} influx and deplete extracellular Ca^{2+} in the intercellular space between apically apposed trophoblast and epithelial cells. As a result, Ca^{2+}-dependent adhesion between epithelial cells (Gumbiner and Simons, 1986) would be disrupted, permitting trophoblastic intrusion of the epithelium. Our data support Yoshinaga's model by providing direct evidence that HB-EGF can induce Ca^{2+} influx in mouse trophoblast cells.

The uterine epithelium is a barrier to interstitial blastocyst implantation (Cowell, 1969), but it is also an important participant in the maternal-embryonic dialogue. During the peri-implantation period, uterine epithelial cells produce HB-EGF (Das et al., 1994) and the uterine glands secrete calcitonin (Ding et al., 1994), two paracrine or juxtacrine agents that are each capable of accelerating blastocyst differentiation. It is interesting that the biological activity of both trophic factors is dependent on Ca^{2+} signaling (Wang et al., 1998 and the present study), which appears to regulate preimplantation embryogenesis beginning as early as the maturing oocyte (Leach et al., 1993; Stachecki et al., 1994a,b; Kono et al., 1996). Therefore, in addition to proximal effects, such as the

proposed downregulation of uterine epithelial cell junctions (Yoshinaga, 1994), HB-EGF-induced signaling promotes the adhesive maturation of trophoblast cells, which is required for subsequent invasion of the decidua. We speculate that the continued and more widespread expression of HB-EGF within the decidua (Das et al., 1994; Leach et al., 1999a) may maintain the adhesive, migratory phenotype of invading trophoblast cells during early placentation.

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