

# Expression of calcitonin receptors in mouse preimplantation embryos and their function in the regulation of blastocyst differentiation by calcitonin

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Accepted 14 August; published on WWW 30 September 1998

## SUMMARY

Calcitonin secretion in the pregnant uterus is tightly regulated by the ovarian hormones, estrogen and progesterone, which limit its expression to a brief period preceding blastocyst implantation. The binding of calcitonin to a G protein-coupled receptor activates adenylate cyclase and elevates cytosolic Ca<sup>2+</sup> levels. The acceleration of preimplantation embryonic development that is known to occur upon elevation of intracellular Ca<sup>2+</sup> prompted an investigation into calcitonin regulation of blastocyst differentiation. Using reverse transcription and the polymerase chain reaction to estimate the relative abundance of calcitonin receptor mRNA, a 25-fold accumulation of the splice variant, CR-1a, was observed in embryos between the 1-cell and 8-cell stages. Cytosolic free Ca<sup>2+</sup> levels were rapidly elevated in embryos at the 4-cell to blastocyst stages after exposure to 10 nM calcitonin.

Blastocysts treated for 30 minutes with 10 nM calcitonin differentiated in vitro at an accelerated rate, as assessed by the translocation of  $\alpha_5\beta_1$  integrin to the apical surface of trophoblast cells, the corresponding elevation of fibronectin-binding activity and the timing of trophoblast cell migration. Chelation of cytosolic free Ca<sup>2+</sup> with BAPTA-AM, but not inhibition of protein kinase A activity by H-89, attenuated the effects of calcitonin on blastocyst development. These findings support the concept that calcitonin secretion within the progesterone-primed uterus and the coordinate expression of CR-1a by preimplantation embryos regulates blastocyst differentiation through receptor-mediated Ca<sup>2+</sup> signaling.

Key words: Blastocyst, Implantation, Calcitonin receptor, Calcium, Integrin, Mouse

## INTRODUCTION

Establishment of pregnancy in mammals is dependent upon successful completion of the embryonic program leading to implantation of the blastocyst in the uterine wall. Bioactive agents produced within the maternal environment regulate embryonic development, as well as uterine receptivity. Several cytokines, including leukocyte inhibitory factor, colony stimulating factor-1, interleukin-1 and heparin-binding EGF-like growth factor, appear to participate in this regulatory process (Pampfer et al., 1991; Das et al., 1994; Stewart, 1994; Simon et al., 1994). Calcitonin, a 32-amino acid polypeptide hormone secreted by the perifollicular C cells of the thyroid (Foster et al., 1964), is up-regulated by progesterone in rat uterine epithelial cells on gestation days 3 and 4, prior to the onset of implantation (Ding et al., 1994, 1995). Calcitonin expression then diminishes as gestation proceeds. Attenuation of uterine calcitonin synthesis during the preimplantation period by administration of calcitonin antisense oligodeoxynucleotides severely impairs rat blastocyst implantation in utero (Zhu et al., 1998). However, the exact role of calcitonin in promoting embryo-uterine interactions is

not clearly understood. Calcitonin regulates Ca<sup>2+</sup> homeostasis in bone and kidney through its interaction with cells possessing calcitonin receptors (Austin and Heath, 1981), which are members of the superfamily of seven transmembrane domain proteins (Goldring et al., 1993; Martin et al., 1995). Several isoforms of the calcitonin receptor have been identified, including two rat isoforms, CR-1a and CR-1b, which differ only by an alternatively spliced, 37-amino acid insertion found in the second extracellular domain of CR-1b (Sexton et al., 1993; Martin et al., 1995).

Human and rodent blastocysts implant interstitially within the uterine decidua (Schlafke and Enders, 1975). Prior to tissue invasion, the mural trophoblast cells undergo a developmental program that culminates with the acquisition of adhesion-competence at their apical surfaces. The trophoblast is initially a polarized epithelium surrounding the newly formed blastocyst (Collins and Fleming, 1995). Trophoblast cells possess a basolateral plasma membrane domain replete with integrins (Hynes, 1992) that mediate trophoblast adhesion to a basal lamina, and an outward-facing apical domain that is largely devoid of integrins (Hierck et al., 1993; Sutherland et al., 1993). As trophoblast differentiation proceeds, adhesion-

promoting integrins translocate to the apical plasma membrane domain (Schultz et al., 1997). Once in vitro cultured blastocysts contact the extracellular matrix (ECM), the trophoblast cells maintain neither their epithelial shape nor tight cell-cell junctions (Enders et al., 1981; Armant et al., 1986a). The morphologically transformed trophoblast then outgrows across the substratum, an experimentally produced phenomenon that is thought to reflect the invasive nature of these cells during implantation in utero (Enders et al., 1981). In vivo, trophoblast invasion of the stroma requires cell adhesion and motility, as well as the ability to secrete proteases that degrade the decidual ECM (Cross et al., 1994).

Schultz and Armant (1995) assayed the fibronectin (FN)-binding activity of intact mouse blastocysts to study integrin-mediated adhesion to the ECM by the apical surface of primary trophoblast cells. Chelators of divalent cations, Arg-Gly-Asp-containing synthetic peptides, or antibodies against the  $\alpha_v$ ,  $\alpha_5$ ,  $\beta_1$  or  $\beta_3$  integrin subunits, all inhibit FN-binding activity, demonstrating that this activity is integrin-mediated (Schultz and Armant, 1995). FN-binding activity becomes maximal 72 hours after initiating serum-free culture of blastocysts collected from the uterus 90 hours post-human chorionic gonadotropin (hCG) injection (Schultz and Armant, 1995; Schultz et al., 1997). Not only is FN-binding activity regulated temporally in accordance with trophoblast outgrowth, but it is also confined to the abembryonic pole of the mouse blastocyst (Schultz and Armant, 1995), which is first to become adhesive in utero and orientates the embryo within the implantation chamber (Kirby et al., 1967). During blastocyst differentiation, adherence to FN by the apical plasma membrane domain of primary trophoblast cells occurs only after the FN-binding integrin,  $\alpha_5\beta_1$ , translocates to the apical domain (Schultz et al., 1997). In addition to the endogenous developmental program that controls trophoblast differentiation to an adhesion-competent stage, contact of the apical cell surface with the ECM directly increases FN-binding activity (Schultz and Armant, 1995). These events do not require new RNA or protein synthesis, but are highly dependent on protein trafficking (Schultz and Armant, 1995).

Intracellular signaling programs the physiological responses of embryonic cells to maternal regulatory factors and, therefore, plays a central role in the maternal-embryonic dialogue. The production of a  $Ca^{2+}$  transient using pharmacological agents such as ethanol or  $Ca^{2+}$  ionophore accelerates the rate of preimplantation development (Stachecki et al., 1994b; Stachecki and Armant, 1996) and facilitates blastocyst differentiation, as indicated by enhanced trophoblast outgrowth in vitro and improved implantation rates after embryo transfer (Stachecki et al., 1994a). Calcitonin increases cAMP levels and induces intracellular  $Ca^{2+}$  oscillations through dual G-protein-coupling of its receptor to adenylate cyclase and phospholipase C (Goldring et al., 1993). Therefore, it is possible that uterine calcitonin secretion prior to implantation stimulates primary trophoblast differentiation through intracellular cAMP or  $Ca^{2+}$  signaling. To examine these possibilities in the mouse, we have documented uterine expression of calcitonin mRNA during early pregnancy and established that mouse blastocysts do indeed express calcitonin receptor mRNA. In addition, we have examined  $Ca^{2+}$  signaling in blastocysts after exposure to calcitonin and the effects of calcitonin on blastocyst differentiation in vitro.

## MATERIALS AND METHODS

### Production of mouse embryos and pregnant uteri

Mouse oocytes and embryos were generated from B6C3F1/J females (Jackson Laboratory, Bar Harbor, ME) that were superovulated by intraperitoneal injection of 5 i.u. pregnant mare serum gonadotropin (Sigma Chemical Company, St Louis, MO) followed 48 hours later by 5 i.u. of hCG (Sigma). To produce embryos, they were mated overnight with males of the same strain immediately after hCG injection. Unfertilized oocytes were collected from non-mated females 14 hours post-hCG by removing the oviduct and releasing the cumulus-oocyte complex through an incision made with a 30-gauge needle in the oviduct wall. Oocytes were freed from cumulus cells by incubation in M2 medium (Sigma) containing 10 mg/ml hyaluronidase (Sigma). Embryos were harvested from pregnant mice at 18, 42, 50, 69 or 90 hours post-hCG injection to obtain the 1-cell, 2-cell, 4-cell, 8-cell or blastocyst stages, respectively. Embryos at the 1-cell to 8-cell stages were flushed from the oviducts with prewarmed M2 medium. Blastocysts were recovered by flushing uterine horns or by culturing embryos collected at the 8-cell stage. The recovered oocytes and embryos were washed free of cumulus and epithelial cells by transfer with a micropipet through several drops of M2 medium containing 4 mg/ml bovine serum albumin (BSA, Sigma). They were then either processed immediately for RNA isolation or cultured in Ham's F-10 medium containing 4 mg/ml BSA, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all from Sigma) at 37°C using a 5%  $CO_2$  incubator. Non-pregnant uteri and uteri from days 1, 2, 3 or 4 of pregnancy were frozen in liquid nitrogen (blastocysts were first flushed from day-4 pregnant uteri).

Some embryos were incubated for 30 minutes or 24 hours in culture medium containing 3-10 nM rat calcitonin (Peninsula Laboratories, Belmont, CA) shortly after harvesting, then washed free of the peptide for continued culture in calcitonin-free medium. Calcitonin was immunoadsorbed by precoating a Petri plate overnight with a 5  $\mu$ l drop of rabbit anti-rat calcitonin antiserum (Peninsula Laboratories) or non-immune rabbit serum (Sigma) under mineral oil at 4°C, washing the drop with culture medium to remove the serum, then incubating medium containing calcitonin with the immobilized antibody. Some blastocysts were incubated for 1 hour in culture medium containing 10  $\mu$ M 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM; Calbiochem, La Jolla, CA) prepared from a 10 mM stock dissolved in dimethylsulfoxide (DMSO). N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl (H-89, Calbiochem) was added to the medium for 90 minutes at 50-250 nM, with some embryos receiving 10 nM calcitonin simultaneously during the period from 30 to 60 minutes. Calcitonin, BAPTA-AM and H-89 were removed before further culture by transferring embryos through several drops of fresh culture medium.

### Trophoblast adhesion assays

Domain analysis of FN demonstrates that only the central cell-binding domain (FN-120; Pierschbacher et al., 1981), through its Arg-Gly-Asp site, is responsible for trophoblast adhesion to FN (Yelian et al., 1995). Therefore, assays for FN-binding activity and blastocyst outgrowth were conducted using FN-120 (Life Technologies, Inc., Gaithersburg, MD), rather than whole FN. Blastocyst outgrowths were produced in serum-free culture medium on plates precoated with 50  $\mu$ g/ml of FN-120, as previously described (Yelian et al., 1995). FN-binding activity was quantified as previously detailed (Schultz and Armant, 1995) using intact blastocysts after culture for 48-72 hours on BSA-coated plates. Prior to assay, the embryos were exposed for 1 hour to 50  $\mu$ g/ml FN-120 to up-regulate adhesion on the apical surface of trophoblast cells.

### Confocal immunofluorescence microscopy

Integrins were visualized on the surface of mouse blastocysts using indirect immunofluorescent antibody labeling and scanning laser

confocal microscopy, as previously described (Schultz et al., 1997). Purified rat monoclonal antibodies against the extracellular domains of the mouse integrin subunits,  $\alpha_5$  (5H10-27) and  $\beta_1$  (9EG7), were purchased from PharMingen (San Diego, CA). Non-immune rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a primary antibody control. Primary antibodies were detected using Texas Red-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories) and nuclei were visualized with 4',6-diamidino-2-phenylindole HCl (DAPI; Calbiochem). Embryos were viewed in a Zeiss (Thornwood, NY) 310 scanning laser confocal microscope at 540 nm for Texas Red staining and at 480 nm for DAPI. Identical contrast and gain settings were used to generate all images, which were saved on an optical disk (Ten X Technologies, Austin, TX), processed using a Power Mac 9500 computer (Apple Computer Corp., Cupertino, CA) and printed using a Tektronix (Wilsonville, OR) Phaser IIS dye-sublimation color printer.

### Intracellular $\text{Ca}^{2+}$

Intracellular  $\text{Ca}^{2+}$  was monitored by fluorescence microscopy in mouse blastocysts incubated for 1 hour in culture medium containing 5  $\mu\text{M}$  fluo-3 acetoxymethyl ester (fluo-3 AM; Molecular Probes, Inc., Eugene, OR), according to Stachecki et al. (1994b). Embryos were cultured during these experiments in Ham's F-10 medium maintained at 37°C using a digitally controlled stage warmer (Brook Industries, Lake Villa, IL). Prior to use, each blastocyst was drawn through a micropipette with a bore measuring two-thirds of the embryo diameter to collapse the blastocoel, which strongly fluoresced and interfered with intracellular  $\text{Ca}^{2+}$  measurements.

To monitor  $\text{Ca}^{2+}$  transients, blastocysts treated with fluo-3 AM were briefly illuminated at 5-second intervals for fluorescence evaluation. Fluorescent images were enhanced with a GenIISys image intensifier (DAGE-MTI, Inc., Michigan City, IN), videotaped and analyzed using a computer-based image analysis system (MCID M4, Imaging Research, St. Catharines, Ontario, Canada). To estimate the concentration of free intracellular  $\text{Ca}^{2+}$ , fluorescent video images were digitized over 16 frames and the area corresponding to an embryo was delineated with a computer mouse and converted to an average fluorescence intensity value by the imaging software.  $\text{Ca}^{2+}$  concentration was estimated from the fluo-3 fluorescence intensity, as previously detailed (Stachecki and Armant, 1996). The  $\text{Ca}^{2+}$  imaging data presented depicts individual embryos that were representative of a minimum of 12 embryos per treatment.

### Reverse transcription and the polymerase chain reaction (RT-PCR)

Unfertilized oocytes and preimplantation embryos were processed for total RNA isolation by the method of Chomczynski and Sacchi (1987). Groups of 100 were transferred through a drop of sterile PBS, immediately lysed in 100  $\mu\text{l}$  4 M guanadinium thiocyanate (Fluka Chemika Biochemika) containing 1 M  $\beta$ -mercaptoethanol (Sigma), then supplemented with 10  $\mu\text{g}$  carrier t-RNA (baker's yeast, Sigma) and 12.5  $\mu\text{g}$  rabbit  $\alpha$ -globin mRNA (Sigma). Mouse uteri were frozen in liquid nitrogen and processed to isolate total RNA using Trizol Reagent (Life Technologies), according to the manufacturer's instructions. The concentration of uterine RNA was determined by the absorbance at 260 nm. Contaminating DNA in all RNA preparations was digested by incubation for 30 minutes at 22°C with 1 unit amplification-grade DNase I (Life Technologies) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM  $\text{MgCl}_2$  and 40 units of RNasin (Promega). Complementary DNA was prepared by reverse transcription from each group of embryos or oocytes, and from 100 ng of uterine RNA. Total RNA was reverse transcribed at 42°C for 1 hour in 20  $\mu\text{l}$  containing 200 units of Superscript II RNase H<sup>-</sup> reverse transcriptase (Life Technologies), 0.25  $\mu\text{M}$  oligo(dT)<sub>12-18</sub> (Life Technologies), 50 mM Tris-HCl, pH 8.3, 140 mM KCl, 2.5 mM  $\text{MgCl}_2$ , 4 mM dithiothreitol, 0.5 units RNasin, and 4  $\mu\text{M}$  of a deoxynucleotide mixture containing dATP, dCTP, dGTP and dTTP

(dNTP; Life Technologies). The reaction was terminated by heating at 95°C for 5 minutes.

$\beta$ -actin cDNA was amplified by the method of DeSousa et al. (1993) to produce a 243-bp amplicon. The  $\beta$ -actin primers generate a larger PCR product from genomic DNA due to the presence of an intron, providing assurance that genomic DNA was absent in all RNA samples. The internal standard,  $\alpha$ -globin, was amplified by the method of Temeles et al. (1994) to produce a product of 257 bp. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was amplified using the primer sequences and PCR conditions of Daston et al. (1996).

Primer sequences for calcitonin (sense: 5' TCAGCATCTTGCTCTGT 3'; antisense: 5' TTGGCCACATCC-CTTTTCTTGC 3') were derived from the published cDNA sequence (Rehli et al., 1996). PCR amplification was carried out in the presence of 1.5  $\mu\text{M}$  of each oligonucleotide primer, 0.5 units of Taq polymerase (Life Technologies), 0.2 mM dNTP, 2.5 mM  $\text{MgCl}_2$ , 50 mM KCl and 50 mM Tris-HCl, pH 8.3. The thermal cycling program consisted of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 1 minute, with a final extension of 9 minutes during the last cycle. An amplicon of 337 bp was generated, as expected, isolated from the agarose gel using a QIAEX gel extraction kit (QIAGEN Ltd., Chatsworth, CA) and incubated for 1 hour at 37°C with *RsaI*, *BsII* or *NlaIV* restriction enzymes (New England Biolabs, Inc., Beverly, MA). Electrophoresis in a 2% agarose gel and ethidium bromide staining revealed restriction fragments of the correct sizes (data not shown).

Calcitonin receptor cDNA was amplified in a modified rat PCR procedure (Firsov et al., 1995) using oligonucleotide primers (sense: 5' TGGTTGAGGTTGTGCCCAATGGA 3'; antisense: 5' CTCGTGGGTTTGCCTCATCTTGGTC 3') derived from the published mouse cDNA sequence (Yamin et al., 1994). These primers generated a product of 503 bp from the CR-1b mRNA, and a product of 392 bp from the CR-1a mRNA, based on rat alternative splicing patterns (Sexton et al., 1993) that appear to be similar in mouse (Ikegame et al., 1995). The reaction mixture for PCR amplification of calcitonin receptor contained 1.5  $\mu\text{M}$  of each oligonucleotide primer, 0.5 units Taq polymerase, 0.2 mM dNTP, 50 mM KCl, 1.5 mM  $\text{MgCl}_2$  and 20 mM Tris-HCl, pH 8.3. The thermal cycling program was identical to that for calcitonin, except the annealing temperature was 62°C. Calcitonin receptor,  $\beta$ -actin and G3PDH amplicons were purified after 40 PCR cycles by agarose gel electrophoresis and a QIAEX gel extraction kit, cloned into a pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced at the Wayne State University macromolecular core facility to confirm their identity.

Relative abundance-reverse transcription and the polymerase chain reaction (RA-RT-PCR) were used to estimate the levels of mRNA for calcitonin, calcitonin receptor and G3PDH, using an approach similar to previous reports (Temeles et al., 1994; Rout et al., 1997; De Sousa et al., 1998). RA-RT-PCR was carried out in 20  $\mu\text{l}$  of reaction mixture containing 1.0  $\mu\text{Ci}$  [ $\alpha$ -<sup>32</sup>P]dCTP (6000 Ci/mmol, Amersham) and cDNA generated from 100 ng of total uterine RNA or an amount of cDNA equivalent to that from a 0.4 part of an embryo or oocyte. Linear incorporation of radioactivity was achieved using 35, 32, 40 or 30 PCR cycles to assay mRNA levels of  $\alpha$ -globin, G3PDH, calcitonin or calcitonin receptor, respectively. Rabbit  $\alpha$ -globin mRNA (0.125 pg/embryo) added to every RNA preparation was used as an internal standard to control for variation between samples in RNA isolation and PCR efficiency (Temeles et al., 1994). PCR product (10  $\mu\text{l}$ ) was mixed with unlabeled product that had been amplified separately for 40 cycles in order to visualize the products after 2.5% agarose electrophoresis and ethidium bromide staining. Amplicons of the expected size were excised under ultraviolet light and subjected to scintillation counting. The relative abundance of calcitonin, calcitonin receptor or G3PDH mRNA was determined during development by normalizing the radioactivity incorporated into each amplicon to the radioactivity incorporated into the  $\alpha$ -globin product from the corresponding sample (Temeles et al., 1994; De Sousa et al., 1998).

### Statistical analysis

All experiments subjected to statistical analysis were repeated at least three times. Percentage outgrowth values were based on a minimum of 90 embryos. Probit analysis was used to compute  $T_{50}$  values (time at which 50% of the blastocysts have commenced outgrowth) and to statistically compare outgrowth rate (Stachecki et al., 1994a). Values reported for FN-binding activity were obtained using at least 15 blastocysts. Differences between treatment groups in the temporal profiles of FN-binding activity were tested for significance using a factorial ANOVA.

## RESULTS

### Calcitonin expression in the pregnant mouse uterus

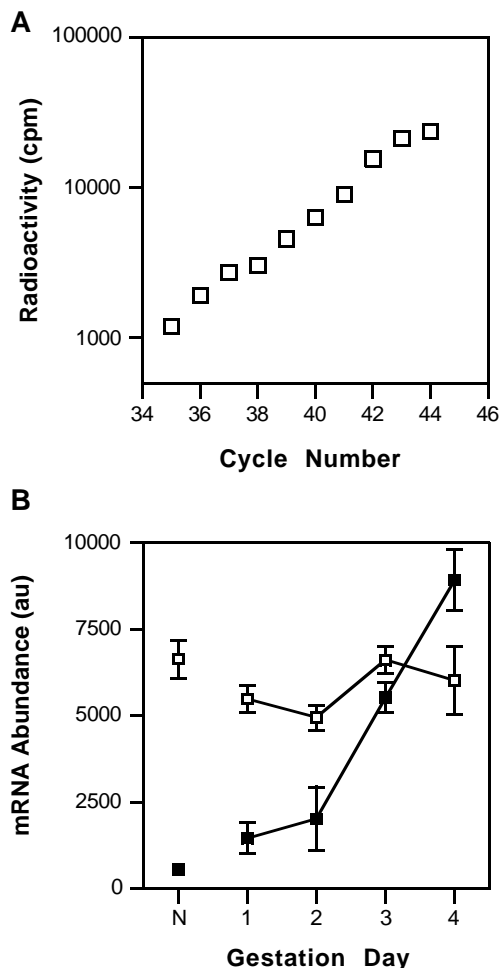
To confirm that calcitonin expression is developmentally regulated in the mouse uterus, as it is in the rat, the relative abundance of calcitonin mRNA was estimated by RA-RT-PCR in non-pregnant and early gestation-stage mouse uteri. Incorporation of radiolabeled dCTP into the calcitonin amplicon was linear between 35 and 42 PCR cycles (Fig. 1A). Calcitonin mRNA increased dramatically between gestation days 2 and 3, as in the rat (Ding et al., 1994), and continued to climb on the fourth day (Fig. 1B). Non-pregnant uteri possessed levels of calcitonin mRNA that were approximately 16-fold lower than gestation day-4 uteri. In contrast to calcitonin transcripts, the levels of G3PDH mRNA were comparable in all uterine tissues. These data suggest that calcitonin accumulates in the mouse uterus during the period prior to blastocyst implantation.

### Calcitonin receptor expression in preimplantation embryos

Only one splice variant of the calcitonin receptor, CR-1a, was detected in preimplantation embryos by RT-PCR (Fig. 2A). CR-1a has been detected previously by RT-PCR in mouse osteoclast cells (Ikegame et al., 1995). Sequencing of the cloned 392 bp PCR product (Accession number AF056329) revealed 99.7% identity with the contiguous portion of the published mouse brain CR-1b sequence (U18542) and the presence of the identical splice site (Houssami et al., 1994; Yamin et al., 1994). RT-PCR amplification of embryonic  $\beta$ -actin mRNA produced a single product of 243 bp, indicating that the RNA preparations were free of genomic DNA (Fig. 2A). The relative abundance of CR-1a mRNA was estimated throughout preimplantation development by RA-RT-PCR. Because the CR-1a PCR reaction was linear between 25 and 40 thermal cycles (Fig. 2B), quantitative RA-RT-PCR was conducted using 30 PCR cycles. CR-1a mRNA was present at a very low level in unfertilized oocytes and zygotes, but increased sharply at the 8-cell stage and continued to accumulate in blastocysts (Fig. 2C). Between the 2-cell and 8-cell stages the relative abundance of CR-1a mRNA increased approximately fourfold. These stages occur on gestation days 2 and 3, respectively, when uterine calcitonin expression first increased. G3PDH mRNA levels remained relatively constant throughout development.

### Ca<sup>2+</sup> signaling in response to calcitonin

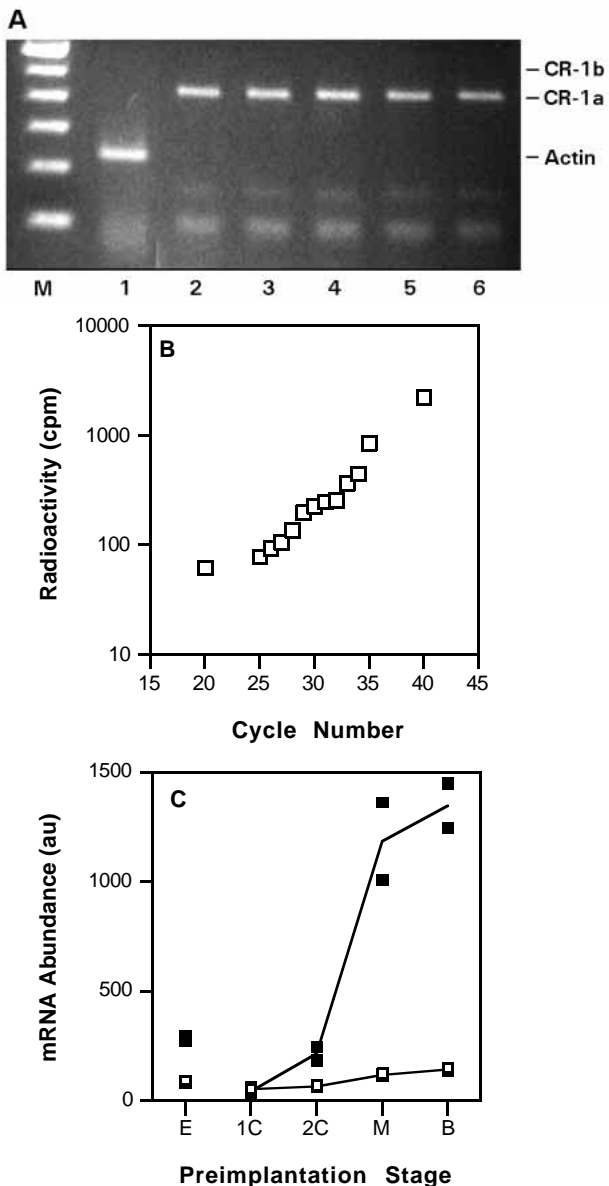
Having established the presence of a developmentally regulated calcitonin receptor in mouse blastocysts, the ability of calcitonin to induce Ca<sup>2+</sup> signaling in blastocysts was next



**Fig. 1.** Expression of calcitonin mRNA in the mouse uterus. RT-PCR was carried out using 100 ng of total RNA isolated from mouse uteri, as detailed in the Materials and methods. (A) Incorporation of [ $\alpha$ -<sup>32</sup>P] dCTP into the calcitonin amplicon after 35 to 44 thermal cycles of PCR, showing the linear range of the reaction. (B) RA-RT-PCR was carried out with RNA from non-pregnant uteri (N) and uteri obtained during pregnancy on gestation days 1-4, as indicated, to estimate the relative abundance (shown in arbitrary units, au) of calcitonin mRNA (■) and G3PDH mRNA (□). Each value is the mean  $\pm$  s.e.m. of four tissue samples.

investigated. Addition of 10 nM calcitonin to blastocysts treated with the fluorescent Ca<sup>2+</sup> indicator, fluo-3-AM, elevated intracellular Ca<sup>2+</sup> levels throughout the embryos (Fig. 3). The effect of calcitonin on intracellular Ca<sup>2+</sup> was rapid and dose-dependent. No change in the intracellular Ca<sup>2+</sup> level was produced by 3 nM calcitonin, and 5 nM was less effective than 10 nM (data not shown). When 10 nM calcitonin was added to the culture medium, the intracellular Ca<sup>2+</sup> concentration in blastocysts rose immediately from approximately 200 nM to over 1  $\mu$ M (Fig. 4), well above the threshold (365 nM) for acceleration of preimplantation development (Stachecki and Armant, 1996). Embryo exposure to calcitonin was followed by a series of Ca<sup>2+</sup> bursts that persisted for at least 5 minutes. No change in Ca<sup>2+</sup> was observed when vehicle alone (culture medium) was added to the embryos. Pre-loading blastocysts with the Ca<sup>2+</sup> chelator, BAPTA-AM, prevented the increased





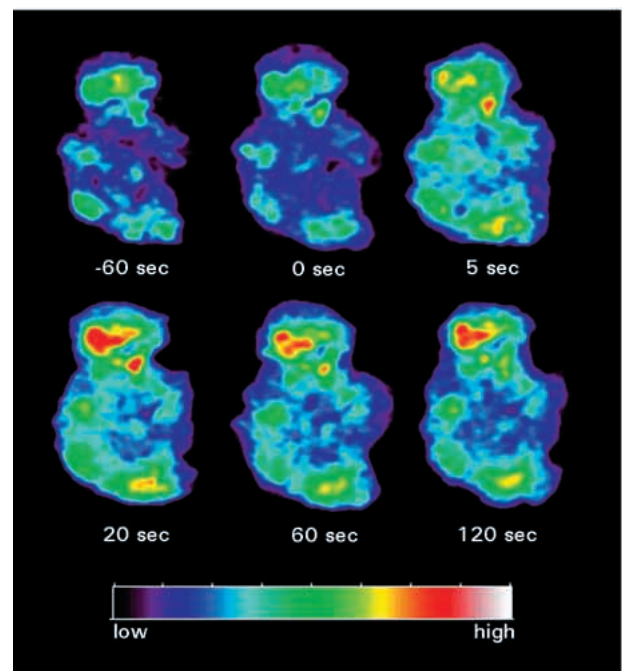
**Fig. 2.** Expression of calcitonin receptor mRNA by developing mouse embryos. (A) 40 cycles of PCR amplification of calcitonin receptor cDNA produced a single 392 bp band corresponding to CR-1a at the blastocyst (lane 2), 8-cell stage (lane 3), 2-cell (lane 4), 1-cell (lane 5) and the unfertilized oocyte (lane 6) stages of development. The 503 bp splice variant, CR-1b, was not detected at any stage. Amplification of  $\beta$ -actin (lane 1) from purified blastocyst RNA by RT-PCR produced a single band of 243 bp, representing the mRNA product. A lane (M) loaded with a 100 bp DNA ladder is shown for reference. (B) Incorporation of [ $\alpha$ - $^{32}$ P]dCTP into the CR-1a amplicon after 20–40 thermal cycles of PCR with 0.4 blastocyst equivalents of cDNA, showing the linear range of the reaction. A similar analysis of the G3PDH reaction produced linear incorporation of radioactivity over the same range of PCR cycles (data not shown). (C) CR-1a (■) and G3PDH (□) mRNA levels were estimated by RA-RT-PCR during preimplantation development at the unfertilized oocyte (E), 1-cell (1C), 2-cell (2C), 8-cell morula (M) and blastocyst (B) stages. The radioactivity incorporated into each amplicon after 30 PCR cycles was normalized to that of  $\alpha$ -globin mRNA assayed in the corresponding sample as an internal standard. Results are shown in arbitrary units (au) for duplicate RNA preparations from each stage.

fluorescence after calcitonin addition, demonstrating that the change in fluorescence is due to a change in  $\text{Ca}^{2+}$  levels.

The response of embryos to calcitonin was monitored using fluo-3-AM at earlier embryonic stages (Fig. 4), since CR-1a mRNA was detectable throughout preimplantation development. The earliest stage that produced a  $\text{Ca}^{2+}$  transient after calcitonin exposure was the 4-cell embryo. Consistent with the relatively low abundance of CR-1a mRNA through the 2-cell stage, 2-cell embryos did not appear to have a functional calcitonin receptor.  $\text{Ca}^{2+}$  transients produced with 10 nM calcitonin in 4-cell, 8-cell and blastocyst-stage embryos were comparable in magnitude.

### Blastocyst development after exposure to calcitonin

Prior research suggests a relationship between induced intracellular  $\text{Ca}^{2+}$  transients and rapid trophoblast outgrowth, as well as improved implantation in utero (Stachecki et al., 1994a). The relationship between  $\text{Ca}^{2+}$  signaling and hormonal stimulation of peri-implantation development is therefore of interest. Immediately after harvest at 90 hours post-hCG injection, blastocysts were exposed for 30 minutes to medium containing 10 nM calcitonin and then cultured to produce trophoblast outgrowths on FN-120-coated plates. Blastocysts exposed to calcitonin outgrew more rapidly than non-exposed embryos (Fig. 5). Probit analysis of the outgrowth curves indicated that the  $T_{50}$  for calcitonin-exposed embryos (62.3 hours) was earlier ( $P < 0.05$ ) than that of control embryos (68.7



**Fig. 3.** Intracellular  $\text{Ca}^{2+}$  signaling in response to calcitonin. Blastocysts were collected at 90 hours post-hCG injection and loaded with fluo-3-AM to monitor the concentration of intracellular  $\text{Ca}^{2+}$ . The relative level of intracellular  $\text{Ca}^{2+}$  was gauged by the fluorescence intensity, which is displayed in pseudocolor, as indicated by the color bar. Baseline levels of intracellular  $\text{Ca}^{2+}$  are shown (–60 sec, 0 sec) in a representative blastocyst. At 0 seconds, 10 nM calcitonin was added to the medium, rapidly increasing intracellular  $\text{Ca}^{2+}$  levels throughout the embryo within 5 seconds. After 120 seconds, the fluorescence began to decline.

hours). Increasing the calcitonin exposure time to 24 hours did not produce additional stimulation (data not shown). This result was similar to the accelerated rate of outgrowth observed after the induction of a  $\text{Ca}^{2+}$  transient in blastocysts by other means (Stachecki et al., 1994a).

Since trophoblast outgrowth requires both embryonic differentiation to the adhesive stage and cell migration, it is difficult to distinguish between rates of blastocyst development using this method. Therefore, FN-binding activity on the blastocyst surface was assessed to gauge more precisely the rate of trophoblast differentiation during *in vitro* culture. Control embryos maximally expressed FN-binding activity after 72 hours (Fig. 6), as previously found (Schultz and Armant, 1995; Schultz et al., 1997). However, FN-binding activity peaked after only 48 hours of culture when the blastocysts were exposed for 30 minutes to 10 nM calcitonin at the outset of culture. The down-regulation of FN-binding activity normally observed after 96 hours of blastocyst culture (Schultz et al., 1997) also occurred precociously at 72 hours in calcitonin-treated embryos. The resulting reversal in the pattern of FN-binding activity at 48 and 72 hours was significant when compared using a factorial ANOVA. As in the outgrowth experiments, longer treatment with calcitonin did not noticeably increase the acceleration of development rate (data not shown). Exposure to calcitonin *in utero* may influence differentiation of blastocysts harvested from the uterus. Therefore, the effect of 10 nM calcitonin was determined using 8-cell embryos flushed from the oviduct, which does not express calcitonin (L. J. Zhu and I. C. Bagchi, unpublished result), after they were cultured to the blastocyst stage. Those blastocysts expressed elevated FN-binding activity at 48 hours (data not shown), similar to the results shown in Fig. 6A.

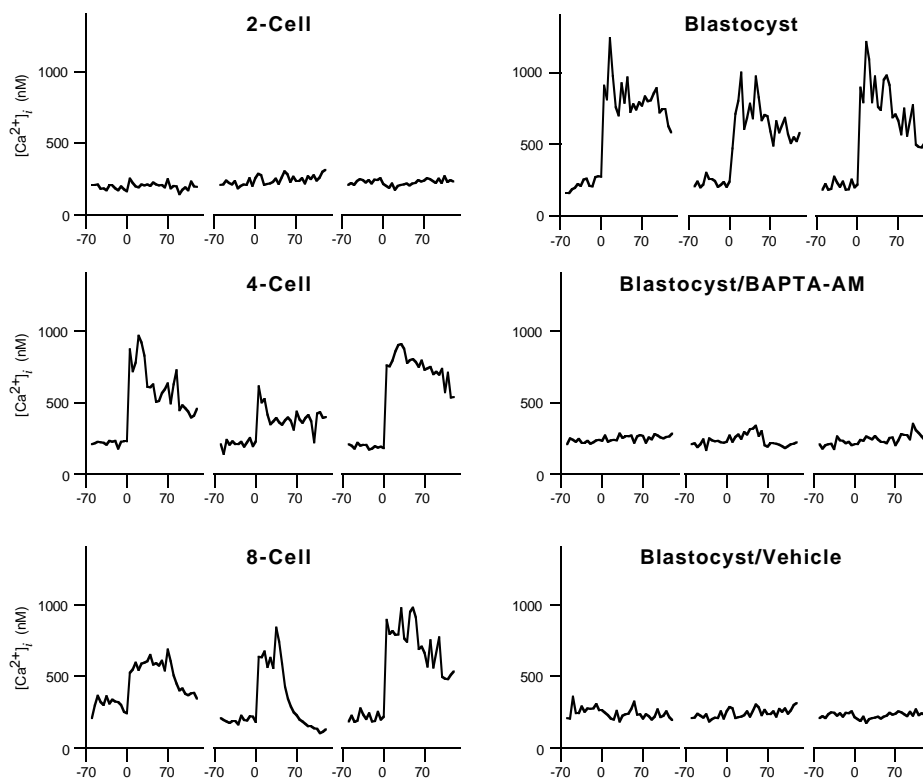
The effect of calcitonin on trophoblast differentiation was dose-dependent. There was no accelerated rate of development when the calcitonin concentration during treatment was reduced to 3 nM, while 5 nM calcitonin accelerated the rate less dramatically than 10 nM (Fig. 6A). Specific pre-adsorption of calcitonin with an immobilized antibody against calcitonin resulted in the restoration of low FN-binding activity at 48 hours, while exposure to immobilized non-immune serum had no effect on the ability of calcitonin to accelerate blastocyst differentiation (Fig. 6B). The neutralizing effect of antibody appeared to be dose-dependent.

The ability of calcitonin to stimulate blastocyst differentiation was further indicated by the early translocation of  $\alpha_5\beta_1$  integrin to the

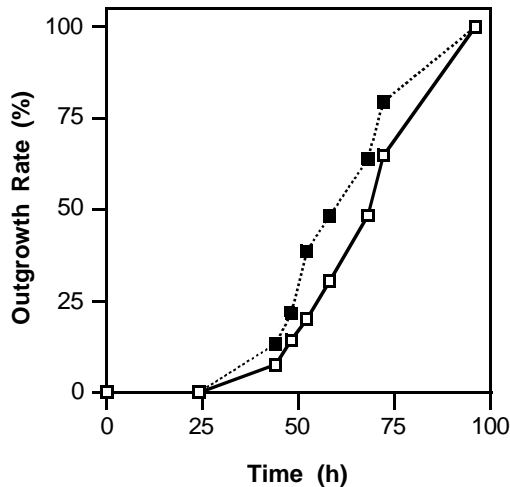
apical surface of trophoblast cells. FN-binding activity on the apical surface of trophoblast cells appears to be mediated by integrins (Schultz and Armant, 1995). Although  $\alpha_5\beta_1$ , which binds to FN, is not present on the apical surface of blastocysts cultured for 48 hours, both subunits appear on the surface after 72 hours, when FN-binding activity is maximal (Schultz et al., 1997). Therefore, the temporal appearance of  $\alpha_5\beta_1$  on the blastocyst surface provides a putative molecular marker of late blastocyst differentiation. Immunohistochemical analysis of the  $\alpha_5$  (Fig. 7A) and  $\beta_1$  (data not shown) integrin subunits on the surface of intact, non-permeabilized blastocysts revealed no staining after 48 hours of culture in control embryos, as expected. However, calcitonin-treated blastocysts expressed  $\alpha_5\beta_1$  on their surface at 48 hours of development (Fig. 7B). Similarly treated blastocysts were not labeled with non-immune IgG (Fig. 7C). The appearance of  $\alpha_5\beta_1$  on the apical surface of trophoblast cells is consistent with the onset of FN-binding activity (Schultz et al., 1997), and the coordinated regulation of both by calcitonin further substantiates this relationship.

### Calcitonin-induced $\text{Ca}^{2+}$ signaling and accelerated blastocyst development

The direct linkage between calcitonin-induced  $\text{Ca}^{2+}$  signaling



**Fig. 4.** Developmental regulation of the intracellular  $\text{Ca}^{2+}$  response to calcitonin. The fluo-3 fluorescence intensity averaged over whole embryos was used to calculate calcitonin-induced changes in the concentration of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) at the 2-cell, 4-cell, 8-cell and blastocyst stages, as indicated. Each graph depicts an individual embryo that was monitored at baseline for 70 seconds ( $-70$ ), exposed at 0 seconds to 10 nM calcitonin or medium without calcitonin (vehicle), and monitored for an additional 120 seconds. In some experiments, blastocysts were loaded with 10 M BAPTA-AM prior to assay (Blastocyst/BAPTA-AM). In addition to the embryos shown here, at least nine other embryos in each treatment group produced similar responses.

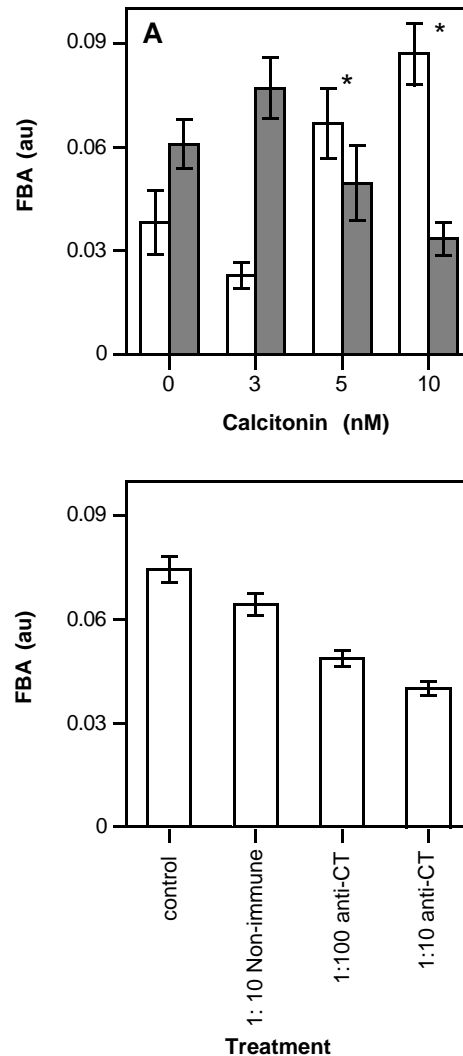


**Fig. 5.** Effect of calcitonin on trophoblast outgrowth in vitro. Blastocysts collected 90 hours post-hCG injection were cultured in vitro for 120 hours on Petri plates coated with 50  $\mu\text{g}/\text{ml}$  FN-120. At the outset of culture, 0 nM ( $\square$ ) or 10 nM ( $\blacksquare$ ) of calcitonin was added to the culture medium for 30 minutes, then removed prior to subsequent culture. Embryos were periodically monitored for the percentage that had initiated trophoblast outgrowth (Armant et al., 1986b; Yelian et al., 1995). The percentages shown represent a total of 90-100 embryos from three separate experiments.

and the ability of calcitonin to accelerate blastocyst development was established using the intracellular  $\text{Ca}^{2+}$  chelator, BAPTA-AM. We have previously shown that preimplantation embryos treated with 10  $\mu\text{M}$  BAPTA-AM are unable to significantly raise their intracellular  $\text{Ca}^{2+}$  levels for at least 2 hours (Stachecki and Armant, 1996). When blastocysts were treated with 10  $\mu\text{M}$  BAPTA-AM for 1 hour before exposure to 10 nM calcitonin, no accelerated rate of trophoblast differentiation was detected, as assessed after 48 hours of culture by the lack of  $\alpha_5\beta_1$  integrin on the apical surface of trophoblast cells (Fig. 7D) and low FN-binding activity (Fig. 8). Since calcitonin also increases intracellular levels of cAMP (Goldring et al., 1993), we examined the effect of H-89, a protein kinase A inhibitor, on calcitonin-treated blastocysts. Blastocysts treated with 50-250 nM H-89 prior to and during calcitonin exposure continued to express elevated FN-binding activity after 48 hours of culture (Fig. 8). H-89 treatment alone had no detectable effect on the developmental regulation of FN-binding activity. We conclude that the ability of calcitonin to stimulate the rate of trophoblast differentiation stems primarily from the associated elevation of cytoplasmic free  $\text{Ca}^{2+}$ .

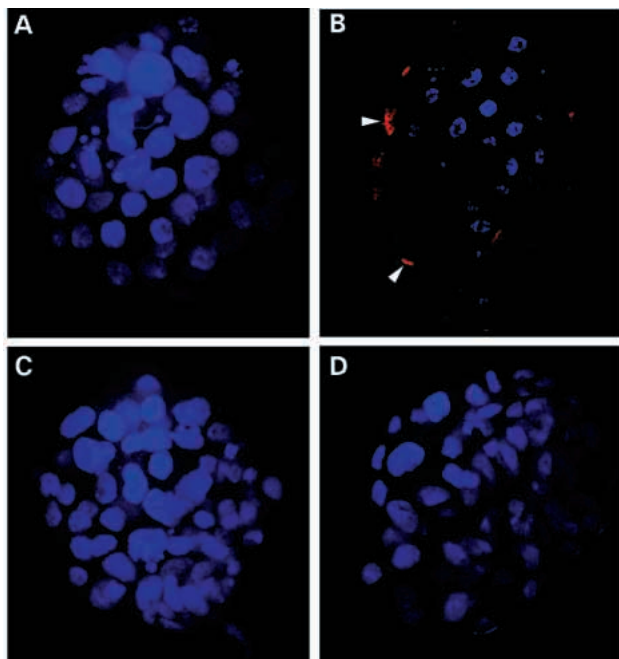
## DISCUSSION

As in the rat (Ding et al., 1994), calcitonin mRNA levels increased dramatically in the mouse uterus between days 2 and 4 of pregnancy. The temporal expression pattern of calcitonin mRNA in the pregnant rat uterus correlates closely with the appearance of immunoreactive calcitonin in the uterine glands (Ding et al., 1994). Steroid hormones strictly regulate uterine calcitonin expression. The onset of uterine calcitonin expression coincides with a rise in serum progesterone level



**Fig. 6.** Effect of calcitonin exposure on the rate of trophoblast differentiation. Blastocysts were removed from culture on BSA-coated plates and assayed for FN-binding activity (FBA), as described in Materials and methods, 48 hours (open bars) or 72 hours (shaded bars) after embryo harvest at 90 hours post-hCG injection. FN-binding activity is expressed in arbitrary units (au) as the mean fluorescence intensity  $\pm$  s.e.m. of at least 15 embryos assayed in three separate experiments. \* $P < 0.05$ , according to factorial ANOVA. (A) Blastocysts were treated with the indicated concentrations of calcitonin for 30 minutes at the outset of culture, as in Fig. 5. (B) Blastocysts were treated with 10 nM calcitonin, as in A, that was untreated (control), pre-adsorbed with 1:10 non-immune rabbit serum (1:10 Non-immune), or pre-adsorbed with 1:10 or 1:100 rabbit anti-rat calcitonin serum (anti-CT).

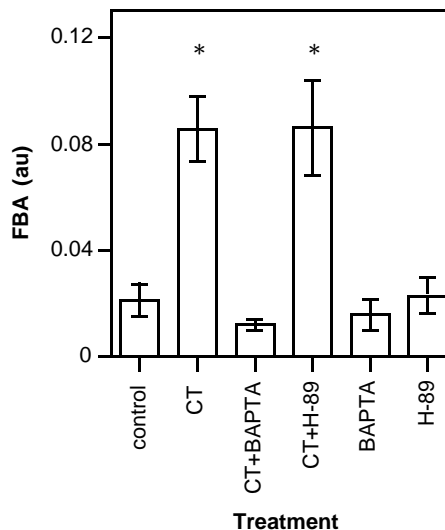
and is abolished by administration of the progesterone receptor antagonist, RU486. Injection of progesterone in rats after ovariectomy increases uterine calcitonin mRNA levels about 20-fold above control animals. Estrogen provides a priming effect when given before progesterone treatments, while it is antagonistic when administered simultaneously with progesterone. In utero administration of calcitonin antisense oligodeoxynucleotides blocks implantation (Zhu et al., 1998), demonstrating that endocrine or paracrine signaling by uterine



**Fig. 7.** Effect of calcitonin exposure on  $\alpha_5\beta_1$  integrin translocation. Blastocysts collected 90 hours post-hCG injection were cultured in vitro on BSA-coated plates. At the outset of culture some embryos were exposed for 30 minutes to 0 nM (A) or 10 nM (B–D) of calcitonin. Some embryos were treated with 10  $\mu$ M BAPTA-AM for 1 hour prior to calcitonin exposure (D). After 48 hours of culture, the embryos were fixed without detergent permeabilization and blastocysts were surface-stained using nonimmune rat IgG (C) or a monoclonal antibody recognizing the integrin  $\alpha_5$  subunit (A,B,D), as described in Materials and methods. Texas Red-conjugated secondary antibody was used to visualize antigen (shown in red) and DAPI was used to identify nuclei (shown in blue). Indirect immunofluorescence staining was visualized by scanning laser confocal microscopy and representative 1  $\mu$ m optical sections are presented here. Antibody staining of  $\alpha_5$  (arrowheads) was only observed in B. Similar results were obtained when an antibody against the  $\beta_1$  subunit was used (data not shown).

calcitonin provides critical stimuli to prepare uterine tissues, the developing preimplantation embryo, or both, for the ensuing events leading to blastocyst implantation.

The observed expression in preimplantation embryos of mRNA for the calcitonin receptor, CR-1a, and its up-regulation during the period of gestation when uterine secretion of calcitonin commences, establishes the embryo as at least one principal target of calcitonin. No expression of the CR-1b isoform was detected by RT-PCR at any stage of preimplantation development. CR-1b is primarily expressed in brain, while CR-1a predominates outside of the central nervous system (Albrandt et al., 1993; Ikegame et al., 1995; Martin et al., 1995; Firsov et al., 1995). The kinetics and pharmacological properties of the two receptors differ, CR-1a having a much higher affinity for calcitonin than CR-1b; however, both receptors are dually coupled to adenylate cyclase and phospholipase C (Albrandt et al., 1993; Houssami et al., 1994; Martin et al., 1995). Therefore, preimplantation embryos expressing CR-1a would be expected to be highly sensitive to calcitonin signaling.



**Fig. 8.** Intracellular  $\text{Ca}^{2+}$  signaling mediates the acceleration of development by calcitonin. FN-binding activity (FBA) was measured after 48 hours of blastocyst culture, as in Fig. 6B. At the outset of culture, blastocysts were exposed for 30 minutes to 0 nM (control) or 10 nM (CT) of calcitonin. Some embryos were exposed to 0 or 10 nM calcitonin after a 1 hour treatment with 10  $\mu$ M BAPTA-AM (BAPTA; CT+BAPTA), or midway during a 90-minute treatment with 250 nM H-89 (H-89; CT+H-89). FN-binding activity is expressed in arbitrary units (au) as the mean fluorescence intensity  $\pm$  s.e.m. of at least 15 embryos assayed in three separate experiments. After 72 hours of culture, embryos treated only with BAPTA-AM or H-89 expressed control levels of FN-binding activity, as seen in Fig. 6A (data not shown). \* $P < 0.05$ , compared to control by ANOVA with a Scheffe post hoc test.

The experiments carried out in this study provide the first direct evidence that calcitonin regulates peri-implantation development through its interaction with embryonic tissues. It is not clear whether the accumulation of CR-1a mRNA during blastocyst formation was the result of transcription or message stabilization. If antibodies against CR-1a become available, it will be important to show that the increased expression of CR-1a mRNA corresponds to increased levels of the receptor protein. However, the ability of calcitonin to induce  $\text{Ca}^{2+}$  transients in blastocysts and accelerate their development outside the uterus, and the ability of an antibody against calcitonin to reverse its activity, strengthen the view that blastocysts possess functional calcitonin receptors. The relative abundance of calcitonin mRNA was low between the oocyte and 2-cell stages and then increased dramatically. Accordingly, intracellular  $\text{Ca}^{2+}$  levels were unaffected by exposure to calcitonin until the 4-cell stage. These data demonstrate that CR-1a becomes active on the surface of blastomeres at the 4-cell stage. Between the 4-cell and blastocyst stages, treatment with 10 nM calcitonin induced a similar increase in the intracellular  $\text{Ca}^{2+}$  concentration, elevating it from 200 nM to 1  $\mu$ M. At the blastocyst stage, the calcitonin dose-dependency for induction of a  $\text{Ca}^{2+}$  transient corresponded with the acceleration of blastocyst differentiation, suggesting a strong relationship between signal transduction in response to calcitonin and regulation of peri-implantation development. The ability of the intracellular  $\text{Ca}^{2+}$  chelator, BAPTA-AM, to prevent the acceleration of trophoblast differentiation by



calcitonin directly linked calcitonin function with an established calcitonin receptor-induced signaling pathway. It is uncertain why in utero exposure to calcitonin prior to 90 hours post-hCG injection did not accelerate blastocyst differentiation relative to naïve blastocysts that were produced by culturing embryos flushed from the oviduct. Blastocysts harvested at 114 hours post-hCG injection acquire FN-binding activity precociously (J. F. Schultz and D. R. Armant, unpublished observation), indicating that later exposure to the uterine environment stimulates blastocyst differentiation. It is not known whether calcitonin concentrations in the uterine lumen exceed 5 nM prior to 90 hours post-hCG injection in mice, so blastocysts collected at that time may be naïve. Alternatively, factors present in the uterine environment prior to 90 hours post-hCG injection may prevent embryos from interacting with or responding to calcitonin.

Calcitonin appears to accelerate blastocyst differentiation by altering the developmental program of the embryo. The  $\text{Ca}^{2+}$  transients produced by calcitonin persisted for only 10-15 minutes (J. Wang and D. R. Armant, unpublished observation) and shifted the onset of FN-binding activity from 72 to 48 hours after treatment. Although the signaling event takes place during a brief period of time, the physiological effects, in terms of integrin trafficking and trophoblast adhesion, were long-range. Calcitonin may reset the developmental 'clock' through  $\text{Ca}^{2+}$  signaling and thereby influence events that occur much later.  $\text{Ca}^{2+}$  signaling rapidly alters gene expression at the blastocyst stage (Rout et al., 1997), probably through downstream activation of transcription factors. The cell cycle is also influenced by  $\text{Ca}^{2+}$  signaling (Lu and Means, 1993), and we have observed increased cell numbers in preimplantation embryos exposed to pharmacological agent that raise the level of intracellular  $\text{Ca}^{2+}$  (Leach et al., 1993; Stachecki and Armant, 1996). Cavitation rates that are altered through manipulation of  $\text{Ca}^{2+}$  signaling correlate with shifts in the rate of cell division (Stachecki and Armant, 1996). It has been suggested that DNA synthesis is directly linked to the timing of developmental events in preimplantation embryos (Spindle et al., 1985; Davis et al., 1996). Replication may provide a window of opportunity for transcription factors to gain access to their cognate cis-binding DNA sequences and thereby reprogram the pattern of subsequent gene expression (Davis and Schultz, 1997). If calcitonin rapidly advances embryonic cells to S phase, subsequent developmental events regulated by the affected transcription factors would be expected to occur precociously.

Ligation of calcitonin to its receptor activates adenylate cyclase and induces intracellular  $\text{Ca}^{2+}$  signaling (Force et al., 1992; Albrandt et al., 1993; Sexton et al., 1993; Houssami et al., 1994). This is particularly interesting in light of reports that monobutyl-cAMP and agents that increase adenylate cyclase activity accelerate the rate of blastocyst cavitation in vitro (Manejwala et al., 1986), and that inhibitors of the cAMP-dependent protein kinase A attenuate these effects (Manejwala and Schultz, 1989). It was unclear whether cAMP levels increased in blastocysts after calcitonin treatment. However, inhibition of protein kinase A with 50–500 nM H-89 ( $K_i = 48$  nM) did not block the ability of calcitonin to accelerate blastocyst differentiation to an adhesive stage. A clear role for cAMP-mediated signaling at this stage or during cavitation has yet to be demonstrated. During cavitation, intracellular concentrations of cAMP do not increase, nor do growth factors

that accelerate cavitation (TGF- $\alpha$ , EGF) increase cAMP levels (Dardik and Schultz, 1992).

Calcitonin-induced  $\text{Ca}^{2+}$  signaling in mouse blastocysts may constitute a critical component of the maternal-embryonic dialogue that regulates early mammalian development. The rate of cavitation associated with blastocyst formation is accelerated by the induction of  $\text{Ca}^{2+}$  transients in compacted morulae on gestation day 3 (Stachecki et al., 1994b; Stachecki and Armant, 1996). Similarly, we have shown here and elsewhere (Stachecki et al., 1994a) that blastocysts collected on gestation day 4 undergo more rapid peri-implantation development after treatment with calcitonin or pharmacological agents that increase the concentration of free cytoplasmic  $\text{Ca}^{2+}$ . Induction of a single  $\text{Ca}^{2+}$  transient is sufficient to accelerate development at the morula or blastocyst stages (Stachecki et al., 1994a,b), perhaps explaining the ability of a 30-minute calcitonin treatment to stimulate blastocyst differentiation as well as exposure for 24 hours. In morulae, acceleration of development after the induction of a  $\text{Ca}^{2+}$  transient is mediated, at least in part, through the activation of calmodulin (Stachecki and Armant, 1996), a  $\text{Ca}^{2+}$ -dependent regulatory protein (Lu and Means, 1993). We have observed a similar dependency on calmodulin during  $\text{Ca}^{2+}$ -mediated acceleration of blastocyst development (L. Mayernik and D. R. Armant, unpublished observation). Therefore, CR-1a signaling through its coupling to phospholipase C and the resultant mobilization of intracellular  $\text{Ca}^{2+}$  may comprise an important biochemical cascade that is responsible for regulating subsequent embryonic development.

This research was supported by National Institutes of Health grant P50AA07606-0010 to D. R. A., a grant from the Department of Obstetrics and Gynecology at Wayne State University and, in part, by center grants P30ES06639 from the National Institute of Environmental Health Science and P30CA22453 from the National Cancer Institute. Support was also provided to I. C. B. by National Institute of Child Health and Human Development grant R01HD34527. We thank Ms Linda Mayernik for assistance with the confocal microscopy experiments and Mr Mike Kruger for his help with the statistical analysis.

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