Expression of the parathyroid Ca^{2+} -sensing receptor in cytotrophoblasts from human term placenta

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Abstract

Fura-2-loaded human cytotrophoblasts responded to elevated extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) with monophasic or, in the case of large ($>20\ \mu\text{m}$) extravillous cells, biphasic elevations in intracellular free Ca^{2+} ion concentration ($[\text{Ca}^{2+}]_i$) that returned to baseline levels after restoration of control $[\text{Ca}^{2+}]_o$. Large extravillous cytotrophoblasts also responded to elevated $[\text{Mg}^{2+}]_o$ with transient elevations in $[\text{Ca}^{2+}]_i$, consistent with the behaviour of the parathyroid Ca^{2+} -sensing receptor. Expression

of the parathyroid Ca^{2+} -sensing receptor in placental cells was confirmed using Northern blot and reverse transcription (RT)-PCR analysis. However, the major transcript in human placental cells (6.2 kb) differed from that expressed by human parathyroid cells (5.6 kb). RT-PCR analysis and DNA sequencing of key PCR products also revealed the presence of a splice variant in placental and parathyroid cells that lacks exon 3.

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Introduction

Recent studies have shown that placental cytotrophoblasts are sensitive to changes in extracellular Ca^{2+} concentration (Juhlin *et al.* 1990, Hellman *et al.* 1992) and that an elevation in extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) suppresses the release of parathyroid hormone-related protein (PTHrP) (Hellman *et al.* 1992). In this respect cytotrophoblasts resemble Ca^{2+} -sensitive chief cells of the parathyroid that are responsible for modulating $[\text{Ca}^{2+}]_o$ via the controlled release of parathyroid hormone. A Ca^{2+} -sensing receptor from human parathyroid cells has recently been cloned, sequenced and expressed in cultured cell lines (Garrett *et al.* 1995a, Bai *et al.* 1996). This protein is a G-protein-coupled receptor (Brown *et al.* 1993) that responds to a variety of divalent and polyvalent cations with elevations in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) via the release of inositol 1,4,5-trisphosphate and is transcribed as a seven exon primary transcript in which the first exon encodes a 5' untranslated region and the last six exons encode the full-length amino acid sequence (Pearce *et al.* 1995). The distribution of this receptor also includes thyroid parafollicular cells, where it appears to regulate calcitonin release (Garrett *et al.* 1995b), renal tubular cells, where it regulates the excretion of Ca^{2+} ions and water (Riccardi *et al.* 1995), the brain (Ruat *et al.* 1995, Ye *et al.* 1996) and antral gastrin cells (Ray *et al.* 1997). However, it is not known whether the parathyroid Ca^{2+} -sensor protein is responsible for Ca^{2+} sensing in

placental cells. In this study we have used a combination of fura-2 microfluorimetry, Northern blot analysis, reverse transcription (RT)-PCR and DNA sequencing to demonstrate that the parathyroid Ca^{2+} -sensor protein is expressed in human placental cells. A splice variant of the receptor that lacks exon 3 of the full length mRNA was identified in both placental and adenomatous parathyroid cells during this study.

Materials and Methods

Cell preparation

Human term placentas were obtained immediately after spontaneous vaginal delivery from either the Royal Hospital for Women, Paddington NSW or King George V Hospital, Camperdown, NSW, Australia. Placental tissue was released with the approval of the hospitals' ethics committees. Approximately 10 g placental tissue was minced and then digested for 30 min at 37 °C in pre-oxygenated physiological saline solution (PSS) that contained collagenase (Worthington type II, 1 mg/ml, Scimar, Templestowe, Victoria, Australia) and DNase I (Sigma Type IV, 0.4 mg/ml, Sigma Chemical Co., St Louis, MO, USA). The composition of PSS was as follows (in mM): NaCl 145, KCl 5.0, H-HEPES 10, CaCl_2 1.0, MgCl_2 1.2, D-glucose 10, Na_2HPO_4 1.0 (pH 7.4 adjusted with NaOH). The cell suspension was filtered, washed by centrifugation and layered on top of a pre-formed Percoll

density gradient (55%) and then centrifuged (480 *g* max for 15 min). Purified cytotrophoblasts were recovered from a band in the density range 1.048–1.062 g/ml as described previously (Bradbury *et al.* 1996). From about 10 g of starting material we obtained $0.8\text{--}2 \times 10^7$ cells.

Human adenomatous parathyroid tissue obtained at surgery (Royal North Shore Hospital, St Leonards, NSW, Australia) was transported to the laboratory immersed in ice-cold PSS. Parathyroid cells were prepared by enzymatic digestion for 30 min at 37 °C in PSS containing 1 mg/ml collagenase (Worthington Type II) and 0.4 mg/ml DNase I (Sigma, Type IV). The suspension was then filtered through nylon mesh (100 µm pore size), washed by centrifugation and resuspended in either PSS for microfluorescence studies (which were performed within 6 h of preparation) or 3 M LiCl/6 M urea solution for the preparation of RNA.

Microfluorescence studies on single cells

Cells were loaded with fura-2 AM (2 µM) for 30 min in RPMI-1640 at 37 °C and then exposed to elevated $[\text{Ca}^{2+}]_o$ as described previously (Bradbury *et al.* 1996). Single cells were excited alternately by light of wavelengths 340 nm and 380 nm at 5 s intervals and the emitted light (peak 510 nm) recorded and stored on computer. The 340 nm/380 nm excitation ratio was used to determine $[\text{Ca}^{2+}]_i$ using a calibration procedure (Bradbury *et al.* 1996).

Preparation of total RNA and mRNA

For the preparation of total RNA, human placental (or parathyroid) cells as described above were resuspended in 2 ml 3 M LiCl and 6 M urea, disrupted by repeated passage through a 21-gauge needle and then sonicated (Auffray & Rougeon 1980). The suspension was incubated overnight on ice and the RNA precipitate was then pelleted by centrifugation. The RNA pellet was further purified by the extraction of protein and DNA with 500 µl phenol:chloroform (25:1) solution (Sambrook *et al.* 1989). mRNA was isolated from 75 µg placental and parathyroid total RNA using Dynabeads mRNA Purification Kit as per the manufacturer's instructions (Dynal, Carlton South, Victoria, Australia).

Preparation of cDNA

Human placental cytotrophoblast and parathyroid cDNA were prepared from 5–10 µg total RNA using Superscript II Reverse Transcriptase at 42 °C for 50 min as per the manufacturer's instructions (Life Technologies, Inc., Mulgrave, Victoria, Australia). RNase H (Boehringer-

Mannheim, Castle Hill, NSW, Australia; 2000 units) was then added for a further 30 min incubation at 37 °C to release single-stranded cDNA.

PCR and DNA sequencing

Primer pairs were chosen using the MacIntosh computer program Amplify according to the published sequence of the human Ca^{2+} -sensitive receptor (Garrett *et al.* 1995a) to generate products that encompassed all six translated exons. The primers (forward, reverse) and their predicted product sizes (in parentheses) were as follows: 5'-CGGGGTACCGCGGCCGCGCCACCATGGGCATT TTATAGCTGC-3' and 5'-GCTCTAGAGCTAGCCC AGGCCTCGCTGG-3' (936 bp) (Bai *et al.* 1996), 5'-GCCCAAAAGAAGGGGGACATT-3' and 5'-AACCA CGTGGAAGTACTGAG-3' (869 bp), 5'-GGAGTAG CAGCTAAAGATCAA-3' and 5'-AACCACGTGGAA GTACTGAG-3' (818 bp), 5'-GCCCAAAAGAAGGG GGACATT-3' and 5'-GAAATACTCGATGATGTC TG-3' (539 bp), 5'-GGAGTAGCAGCTAAAGATCAA-3' and 5'-GAAATACTCGATGATGTCTG-3' (488 bp), 5'-TTCCTGAAGAAGGTCCATCC-3' and 5'-GGCAT AGACGTTGTAATACC-3' (551 bp), 5'-GGACCAG GAAAGGGATCATT-3' and 5'-CGGTTGGTTTTC ACCAGGAT-3' (459 bp), 5'-CTGGCGTGCATCTT CTTCAA-3' and 5'-GTTTTCTGTAACAGTGCTGC 3' (680 bp).

PCR was performed by the addition of primers (2.5 µl) and cDNA (2–6 µl) to 45 µl PCR Supermix (Life Technologies Inc.). The following protocol was used in a Hybaid Omn-E thermal cycler: 94 °C for 30 s, 50 °C for 1 min 30 s, 72 °C for 2 min for 35 cycles then 72 °C for 20 min. PCR products were sequenced as required by a Perkin Elmer Catalyst 800 robotic work station using the dye-terminator method.

Northern blot analysis

Total RNA or mRNA was size fractionated on a 1.0% agarose/formaldehyde gel (Sambrook *et al.* 1989), transferred to a N⁺-Nylon membrane (Amersham Life Science, Castle Hill, NSW, Australia) by Northern blot transfer (Sambrook *et al.* 1989), and UV fixed for 1 min (Bio-Rad GS Gene Linker UV Chamber, Bio-Rad Lab, Hercules, CA, USA). A 680 bp DNA probe was prepared from human placental cDNA by PCR using the following primers: forward, 5'-CTGGCGTGCATCTTCTTCAA-3'; reverse, 5'-GTTTTCTGTAACAGTGCTGC-3'. It was excised from a 3.0% agarose gel (NuSieve GTG Agarose, Edwards Instruments, Narellan, NSW, Australia) after electrophoresis and recovered by centrifugation in a Spin-X tube (Trace Scientific, Clayton, Victoria, Australia). The filtrate containing purified DNA was ³²P-labelled by the random-primer method using [³²P]d-CTP (Rediprime labelling kit, Amersham Life Science)

Figure 1 Response of a fura-2-loaded placental cytotrophoblast to elevated free Ca^{2+} and Mg^{2+} concentrations. A fura-2-loaded cytotrophoblast ($>20\ \mu\text{m}$ diameter) was exposed to elevated $[\text{Ca}^{2+}]_o$ (5.0 mM) and then elevated $[\text{Mg}^{2+}]_o$ (20 mM) in a microfluorescence apparatus. Control $[\text{Ca}^{2+}]_o$ was 1.0 mM and control $[\text{Mg}^{2+}]_o$ was 1.2 mM. Similar data were obtained in three other experiments.

and separated from free $[\text{32P}]\text{dCTP}$ using Pharmacia Nick columns (Pharmacia Biotech, Melbourne, Victoria, Australia).

The N^+ nylon membrane was prehybridized in a 20 ml solution consisting of $5 \times \text{SSPE}$ (NaCl 0.75 M, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.05 M, EDTA 0.005 M), $5 \times \text{Denhardt's}$ solution, 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$ sonicated salmon testis DNA at 50°C for 4 h. The nylon membrane was then hybridized in a 10 ml solution consisting of $5 \times \text{SSC}$ (NaCl 0.75 M, $\text{Na}_3\text{ citrate}$ 0.075 M), 1.25% Denhardt's solution, 30 mM KH_2PO_4 , 0.25% SDS, 50% formamide, 50 mg/ml dextran sulphate, and 100 $\mu\text{g}/\text{ml}$ sonicated salmon testis DNA and 100 μl ^{32}P -labelled DNA probe at 50°C for 18 h. Following hybridization, the blot was washed initially in $2 \times \text{SSC}$, 0.1% SDS at room temperature for 5 min, then two 30-min washes in $2 \times \text{SSC}$, 0.1% SDS at 65°C (low stringency) followed by two 30-min washes in $0.2 \times \text{SSC}$, 0.1% SDS at 65°C (high stringency). The latent ^{32}P -labelled image was captured by an exposure cassette for 24 h and then stored to computer using a PhosphorImager.

Results

Elevated Ca^{2+} - and Mg^{2+} -induced elevations in $[\text{Ca}^{2+}]_i$ in fura-2-loaded human cytotrophoblasts

Single human cytotrophoblasts responded to elevated $[\text{Ca}^{2+}]_o$ from 1.0 to 5.0 mM with monophasic or, in the case of some large cells (diameter $\geq 20\ \mu\text{m}$), biphasic elevations in $[\text{Ca}^{2+}]_i$. Nineteen of twenty-two large cells tested (ten placental preparations) responded to elevated $[\text{Ca}^{2+}]_o$ with elevations in $[\text{Ca}^{2+}]_i$. Ten of these nineteen

Figure 2 RT-PCR analysis of human cytotrophoblast cDNA for parathyroid Ca^{2+} -sensing receptor. Exon-by-exon analysis was performed using either human placental cytotrophoblast cDNA (lanes 1, 3, 5 and 7) or human adenomatous parathyroid cDNA (lanes 2, 4, 6 and 8) as the templates. Based on the published sequence of the human Ca^{2+} -sensing receptor, products of 869 bp (exons 2–4; lanes 1 and 2), 551 bp (exons 4–5; lanes 3 and 4), 459 bp (exons 6–7, lanes 5 and 6) and 680 bp (exon 7; lanes 7 and 8) were expected (see exon map). The additional, smaller products, amplified from cytotrophoblast cDNA (lane 1) and from parathyroid cDNA (lane 2) were found to be identical by DNA sequencing.

Ca^{2+} -sensitive cells responded with biphasic elevations (Fig. 1). Four of eleven large cells also responded to elevated $[\text{Mg}^{2+}]_o$ from 1.2 to 20 mM with transient elevations in $[\text{Ca}^{2+}]_i$ (Fig. 1). This behaviour resembles that observed in human parathyroid cells (Nemeth & Scarpa 1987).

RT-PCR analysis of human cytotrophoblast cDNA for the parathyroid Ca^{2+} -sensing receptor

PCR primers were designed to amplify DNA products from the six exons (exons 2–7) that encode the Ca^{2+} receptor protein. The expected PCR products and their exon(s) of origin were as follows: 869 bp (exons 2–4), 551 bp (exons 4–5), 459 bp (exons 6–7) and 680 bp (exon 7). The major PCR products obtained from human adenomatous parathyroid cDNA using these primer combinations conformed to the expected sizes indicating that all six exons are expressed (Fig. 2). The major PCR products obtained from human cytotrophoblast cDNA using these primers for exons 4–5, 6–7 and 7 also conformed to the expected sizes. However, the major product obtained using the exon 2–4 primers was shortened by approximately 300 bp (Fig. 2).

Northern blot analysis of total RNA and mRNA from human cytotrophoblasts for the expression of the parathyroid Ca^{2+} -sensing receptor

RNA from human cytotrophoblasts was analysed for the expression of the parathyroid Ca^{2+} -sensing receptor using

Figure 3 Northern blot analysis of the parathyroid Ca^{2+} sensor in parathyroid and placental cells. (A) Total RNA from placental cytotrophoblasts (30 μg , lane 1) or adenomatous parathyroid cells (5 μg , lane 2) was separated by agarose electrophoresis and then exposed to a ^{32}P -labelled 680 bp DNA probe for the Ca^{2+} -sensing receptor. (B) mRNA from placental cytotrophoblasts (3 μg , lane 3) or adenomatous parathyroid cells (0.5 μg , lane 4) was separated by agarose electrophoresis and then exposed to the probe described in (A).

a 680 bp ^{32}P -labelled DNA probe. This probe identified transcripts of several different sizes (including 10 kb, 5.6 kb and 4.8 kb) in total RNA from human adenomatous parathyroid cells (Fig. 3); transcripts greater than 4 kb have been previously shown to encode full-length Ca^{2+} receptor proteins (Garrett *et al.* 1995a). The probe also identified transcripts of 6.2 kb, 4.8 kb and 2.2 kb in total RNA from placental cytotrophoblast total RNA (Fig. 3). Using mRNA, major transcripts of 6.2 kb (placenta) and 5.6 kb (parathyroid) were identified.

Analysis of splice variant by RT-PCR and DNA sequencing

To investigate the possibility that exon 3 was missing in an alternative transcript of the receptor in cytotrophoblasts, new primer pairs for exons 2–4 were used. PCR products of the following sizes were predicted from the published parathyroid cDNA sequence: 936, 818, 539 and 488 bp. In this experiment, two major products were generated from cytotrophoblast cDNA using all four primer combinations, one of the expected size i.e. consistent with the expression of a full length transcript and, as before, one shortened by about 300 bp (Fig. 4). DNA sequencing of the shortened PCR products confirmed that exon 3 was missing and that the 3' end of exon 2 was directly spliced (out of frame) to the 5' end of exon 4 (Fig. 5). The sequence obtained indicates that the splice variant encodes a truncated protein of 153 amino acids. PCR analysis of human parathyroid cDNA revealed the presence of a similar splice variant lacking exon 3.

Discussion

The transfer of Ca^{2+} ions across the placenta from mother to fetus is essential for fetal Ca^{2+} homeostasis. The finding that placental cells are Ca^{2+} -sensitive and that elevated

Figure 4 RT-PCR analysis for detection of exon 3 splice variants. Human placental cytotrophoblast cDNA (lanes 1, 3, 5 and 7) and adenomatous parathyroid cDNA (lanes 2, 4, 6 and 8) were used as templates, with forward and reverse primers designed to bind within exons 2 and 4 respectively. Based on the published sequence of the human Ca^{2+} -sensing receptor, products of 936 bp (lanes 1 and 2), 818 bp (lanes 3 and 4), 539 bp (lanes 5 and 6) and 488 bp (lanes 7 and 8) were predicted (see exon map). An exon 3 splice variant was detected as a product shortened by 300 bp in each of the cytotrophoblast lanes (lanes 1, 3, 5 and 7) and to a lesser extent in the parathyroid lanes (lanes 2, 4, 6 and 8).

Figure 5 Sequence analysis of the exon 3-deficient variant of the Ca^{2+} -sensing receptor. Sequences of the 2/3 and 3/4 exon junctions in the native Ca^{2+} -sensing receptor (a) and of the placental 2/4 splice variant (b) are shown. The sequence indicated that the splice variant undergoes a frameshift and that the encoded protein is truncated by the presence of a premature stop codon.

extracellular Ca^{2+} concentration may suppress the local release of the fetal calcitrophic hormone PTHrP (Hellman *et al.* 1992) suggests that the placenta itself has a role in regulating Ca^{2+} transfer. A Ca^{2+} sensor on placental cells has been studied previously. This 500 kDa protein from human placenta (Juhlin *et al.* 1990) has now been cloned (Hjalm *et al.* 1996). However, its role in Ca^{2+} sensing has not yet been confirmed by the expression of the protein in cultured cells.

We pursued an alternative hypothesis in this study – that placental cytotrophoblasts express the cloned parathyroid G-protein-coupled Ca^{2+} -sensing receptor (Brown *et al.* 1995, Garrett *et al.* 1995a). This hypothesis is suggested by the finding that a subpopulation of fura-2-loaded cytotrophoblasts (large cells >20 μm diameter)

exhibited biphasic elevations in $[\text{Ca}^{2+}]_i$ when exposed to elevated $[\text{Ca}^{2+}]_o$ (from 1.0 to 5.0 mM) which took the form of an initial transient peak followed by a sustained plateau response which returned to baseline levels when $[\text{Ca}^{2+}]_o$ returned to control levels. In addition, these cells exhibited transient elevations in $[\text{Ca}^{2+}]_i$ when exposed to elevated $[\text{Mg}^{2+}]_o$ (from 1.2 to 20 mM) (Fig. 1). These data are consistent with the hypothesis that elevated $[\text{Ca}^{2+}]_o$ and $[\text{Mg}^{2+}]_o$ activate a surface receptor coupled to the release of Ca^{2+} ions from an intracellular store as occurs with activation of the parathyroid G-protein-coupled Ca^{2+} -sensing receptor.

Consistent with this hypothesis, Northern blot analysis of RNA from cytotrophoblasts using a ^{32}P -labelled 680 bp DNA probe revealed the presence of several transcripts (Fig. 3). The transcript pattern (6.2 kb, 4.8 kb and 2.2 kb) differed from the pattern observed in total RNA derived from human parathyroid adenomas (10 kb, 5.6 kb and 4.8 kb). Furthermore, when mRNA was used, the major transcripts identified were 6.2 kb from placenta and 5.6 kb from parathyroid (Fig. 3). The pattern of human parathyroid transcripts we observed corresponds closely to that reported previously (Tsai-Morris *et al.* 1990, Garrett *et al.* 1995a). The level of expression of mRNA for the Ca^{2+} -sensing receptor was lower in the placenta than in the parathyroid. This almost certainly reflects the fact that parathyroid cells are largely homogeneous whereas cytotrophoblasts are heterogeneous; only a restricted subpopulation of cytotrophoblasts is likely to express the Ca^{2+} -sensing receptor.

Our data suggest the presence of full length transcripts in both placenta and adenomatous parathyroid, together with a splice variant lacking exon 3. The splice variant encodes a truncated protein of 153 amino acids (compared with 1078 amino acids for the full length protein) and if translated would not be incorporated into the plasma membrane. N-terminally truncated forms of other G-protein-coupled receptors have been described. In the case of the luteinizing hormone receptor, an N-terminally truncated form of the receptor is released into the extracellular fluid of cultured cells that express it and may be released into the circulation *in vivo* (Tsai-Morris *et al.* 1990). It is possible that the truncated form of the Ca^{2+} -sensing receptor we have identified is also released into the circulation but the functional significance of this, if it occurs, is unclear.

Placental cells are heterogeneous. First, there are important differences between *villous* cytotrophoblasts, which lie deep to the syncytial barrier between fetal and maternal circulations, and *extravillous* cytotrophoblasts which are bathed by maternal blood (Douglas & King 1989, Shorter *et al.* 1993). Secondly, there are differences in the differentiation state of extravillous cytotrophoblasts which increases progressively with increasing cell size (Rachmilewitz *et al.* 1993). We have previously characterized three major subpopulations of cytotropho-

blasts using freshly dispersed cells from the human term placenta. Two of these three subpopulations were Ca^{2+} sensitive (Bradbury *et al.* 1996). The smaller of the two Ca^{2+} -sensitive cell types (cell size $\leq 10 \mu\text{m}$) was found to correspond to villous cytotrophoblasts which were negative for cytokeratin-8, vimentin and major histocompatibility complex class I antigens as determined using W6/32 monoclonal antibodies (Bradbury *et al.* 1996). When loaded with fura-2, villous cytotrophoblasts failed to respond to extracellular ATP or UTP. By virtue of their position, villous cytotrophoblasts are exposed to fetal $[\text{Ca}^{2+}]_o$.

The larger of the two Ca^{2+} -sensitive cell types (25–50 μm), which were positive for cytokeratin-8 and negative for vimentin, were found to be extravillous in origin based on the expression of MHC class I antigens (Bradbury *et al.* 1996). Unlike villous cytotrophoblasts, these extravillous cells also responded to extracellular ATP and UTP when loaded with fura-2. By virtue of their position, extravillous cytotrophoblasts are exposed to maternal $[\text{Ca}^{2+}]_o$. Data obtained in the present study indicate that extravillous cytotrophoblasts exhibit biphasic elevations in $[\text{Ca}^{2+}]_i$ in response to elevated $[\text{Ca}^{2+}]_o$ and are also Mg^{2+} sensitive.

Taken together, the data presented here indicate that the parathyroid Ca^{2+} -sensing receptor is expressed in the placenta most probably by extravillous cytotrophoblasts.

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