Biochem. J. (1988) 250, 877–882 (Printed in Great Britain)

Human placental calcitonin receptors

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Receptors for the hypocalcaemic hormone, calcitonin (CT), have been identified in a membrane fraction prepared from term human placentae. Binding of ^{125}I-labelled salmon CT (^{125}I-sCT) to the membranes was time- and temperature-dependent, saturable ($B_{max.}$ 58 ± 11 fmol/mg of protein), of high affinity (K_d 80 ± 21 pM) and poorly reversible. Species-specific CTs and CT analogues competed for ^{125}I-sCT binding with potencies proportional to their known biological potencies. Various unrelated peptide hormones did not compete, indicating that receptor binding was specific for CT. Photoaffinity labelling using a derivatized biologically active sCT analogue, [Arg^{11,18},3-nitrophenylazide-Lys^{14}]sCT, identified a receptor component of $M_r \sim 85000$, comparable with findings in osteoclasts and other target cells. The presence of CT receptors in the human placenta supports other evidence that CT may have a role in the regulation of placental function.

INTRODUCTION

The calcium-lowering property of CT in mammals led to its discovery by Copp and his associates over 25 years ago [1]. Subsequent investigations have shown that the osteoclast [2–5] and renal tubular cells [6–9] are major target cells. Nevertheless, a number of other tissues possess CT receptors, including brain [10–12], lung [13], testes [14], and trout gill [15] as well as cell lines derived from a wide range of neoplasias including human breast [16], lung [17], and renal carcinoma [18], leukaemia [19,20], teratocarcinoma [21] and osteogenic sarcoma [22]. However, none of these has provided a substantial source of receptor because of either low specific binding activity, scarcity of tissue, or both.

The placenta contains receptors for several peptide hormones including insulin [23], epidermal growth factor [24], the somatomedins [25–27] and gonadotrophin releasing hormone [28] and has been used as a source of receptor for the purification of several of these. In this study we have identified, and characterized, CT receptors in a human placental membrane fraction. The presence of CT receptors in the placenta supports other evidence (reviewed in [29,30]) that CT may be important in foeto-maternal calcium metabolism.

MATERIALS AND METHODS

Chemicals and hormones

Synthetic human CT (hCT) was provided by Dr. W. Rittel, Ciba Geigy, Basle, Switzerland. Synthetic salmon CT (sCT), porcine CT (pCT), des-Leu^{16}-sCT, des-Phe^{16}-hCT and [Gly^{8}]hCT were provided by Dr. R. C. Orlowski, Armour Pharmaceutical Co., Kanakee, IL, U.S.A. Aminosuberate eel CT ([Asu$^{1-7}$]eCT) and synthetic human PTH-(1–34) (hPTH) were provided by Dr. J. Murase, Toyo Jozo Co., Tokyo, Japan. The sCT analogue [Arg^{11,18},Lys^{14}]sCT was synthesized on an Applied Biosystems 430A Automatic Peptide Synthesizer, cleaved from the resin with anhydrous HF and purified by reversed-phase h.p.l.c. The [Arg^{11,18},Lys^{14}]-sCT chromatographed as a single peak on reversed-phase h.p.l.c. and had the expected amino acid composition following quantitative amino acid analysis. The derivative [Arg^{11,18},3-nitrophenylazide-Lys^{14}]sCT was prepared from 4-fluoro-3-nitrophenylazide (Pierce Chemical Co.) and purified by reversed-phase h.p.l.c. Both [Arg^{11,18},Lys^{14}]sCT and [Arg^{11,18},3-nitrophenylazide-Lys^{14}]sCT are biologically active (equipotent with native sCT) (C. D'Santos, unpublished work). Synthetic human calcitonin gene-related peptide (hCGRP) was obtained from Bachem Inc. Synthetic ACTH-(1–24) was obtained from Ciba Geigy. GTP and GTPγS were obtained from Boehringer Mannheim. Na^{125}I, [^{32}P]ATP and cyclic [^{3}H]AMP were obtained from The Radiochemical Centre, Amersham. All other chemicals were obtained from standard suppliers.

Preparation of placental membrane fraction

Placentae were placed on ice immediately after delivery and all subsequent procedures were carried out at 4 °C. The placental membrane fraction was prepared as previously described [31], and summarized as follows. Parenchymal tissue was dissected free of chorion, amnion, cord, and large blood vessels. The tissue was chopped, blended and subsequently homogenized in 25 mM-Tris/250 mM-sucrose/5 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride, pH 7.4. The homogenate was centrifuged at 10000 *g* for 30 min. The supernatant was fortified to 100 mM-NaCl and 0.2 mM-$MgSO_4$, and centrifuged at 30000 *g* for 1 h. The pellets were resuspended in 25 mM-Tris/5 mM-EDTA, pH 7.4,

Abbreviations used: CT, calcitonin; the prefixes h, s, p and e refer to the human, salmon, pig and eel hormones respectively; PTH, parathyroid hormone; hCGRP, human calcitonin gene-related peptide; ACTH, adrenocorticotropin; PAGE, polyacrylamide-gel electrophoresis; GTPγS, guanosine 5′-[γ-thio]triphosphate.

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and centrifuged at 30000 *g* for 1 h. This washing step was repeated once more and the membrane fraction was stored in washing buffer at −80 °C. Protein concentrations were assayed by the method of Lowry [32], using bovine serum albumin as the standard.

^{125}I-sCT binding to placental membranes

^{125}I-sCT was prepared using chloramine-T oxidation and QUSO G32 purification [33]. Specific radioactivity was 150–200 μCi/μg. Placental membranes were washed in 50 mM-Tris, pH 7.0, and then resuspended (1–2 mg of membrane protein/ml) in the same buffer with 1 mg of bovine serum albumin/ml (assay buffer). Incubations were performed with ^{125}I-sCT alone or together with unlabelled peptides. Non-specific binding was assessed in the presence of excess unlabelled sCT (300 nM). To terminate the reaction, the incubation mixtures were chilled to 0 °C and 50–150 μl aliquots were layered on 200 μl of chilled assay buffer in microcentrifuge tubes which were then centrifuged at 10000 *g* for 5 min. The pellets were washed once with assay buffer and recovered by the method of Rodbell [34]. Bound radioactivity was counted in a Packard Autogamma γ-counter with 70% efficiency. The data are expressed as means of triplicate determinations. S.E.M. values (not shown) were less than 5% of the mean in all cases.

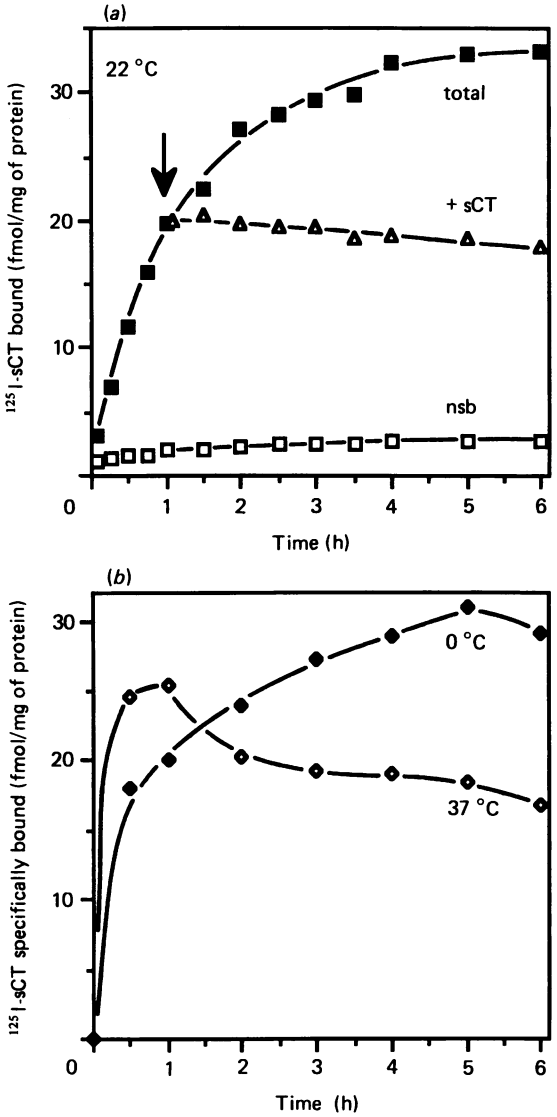

Fig. 1. Time- and temperature-dependency of ^{125}I-sCT binding in placental membranes

(*a*) Placental membranes were incubated at 22 °C with 0.1 nM-^{125}I-sCT alone to measure total binding (total, ■) or together with excess (300 nM) unlabelled sCT to measure non-specific binding (nsb, □). At the indicated times triplicate aliquots were removed for estimation of binding as described in the Materials and methods section. After 1 h, as indicated by the arrow, excess sCT was added to a portion of the 'total' incubation mixture to measure the reversibility of binding (+sCT, △). (*b*) Membranes were incubated at 0 °C (◆) or 37 °C (◇) with 0.1 nM-^{125}I-sCT. Points represent specific binding (total minus non-specific).

Photoaffinity labelling of the CT receptor with ^{125}I-[Arg^{11,18},3-nitrophenylazide-Lys^{14}]sCT

Placental membranes were incubated in the dark in assay buffer containing 0.2 nM-^{125}I-[Arg^{11,18},3-nitrophenylazide-Lys^{14}]sCT alone or together with excess unlabelled sCT for 2h at 22 °C. All subsequent procedures were performed at 4 °C. The membranes were centrifuged at 10000 *g* for 5 min. The pellets were washed in phosphate-buffered saline, resuspended in the same buffer and irradiated for 15 min with a 100 W long wave (peak emission of 366 nm) u.v. lamp. They were solubilized for 30 min at 4 °C in 0.1% Triton X-100 in 50 mM-Tris, pH 8.0, and centrifuged at 100000 *g* for 1 h. Extracts were reduced by boiling for 3 min in 5% (v/v) mercaptoethanol and then analysed by SDS/PAGE by the method of Laemmli [35]; 10% disk gels [10% (w/v) acrylamide and 0.25% (w/v) bisacrylamide] with a 3% stacking gel were employed. Radioactivity was measured in 2 mm slices of the gels. M_r standards were run on parallel gels.

Adenylate cyclase activity

Adenylate cyclase activity was measured in both the placental membrane fraction prepared as described above and in freshly prepared homogenate. The latter was prepared by homogenization of placental tissue in 25 mM-Tris/30 mM-KCl/4.5 mM-$MgSO_4$/0.1 mg of bovine serum albumin/ml, pH 7.6, followed by centrifugation at 2000 *g* for 10 min. Only the white-coloured fraction of the resulting pellet was used. The preparations were incubated with [^{32}P]ATP in an ATP-regenerating system of phosphoenolpyruvate and pyruvate kinase as previously described [36].

RESULTS

Effect of buffer composition, pH and protein concentration on ^{125}I-sCT binding to placental membranes

Maximal specific binding occurred in the range pH 6–7 in either 50 mM-Tris or 100 mM-K_2HPO_4/KH_2PO_4 buffer. Specific binding was not affected by Ca^{2+} (5 and 10 mM), Mg^{2+} (5 and 10 mM) nor EDTA (1 and 5 mM), but was reduced to approx. 60% of control in the presence of NaCl (150 mM) or KCl (150 mM). Specific binding bore a linear relationship to membrane protein concentrations up to 1.2 mg/ml.

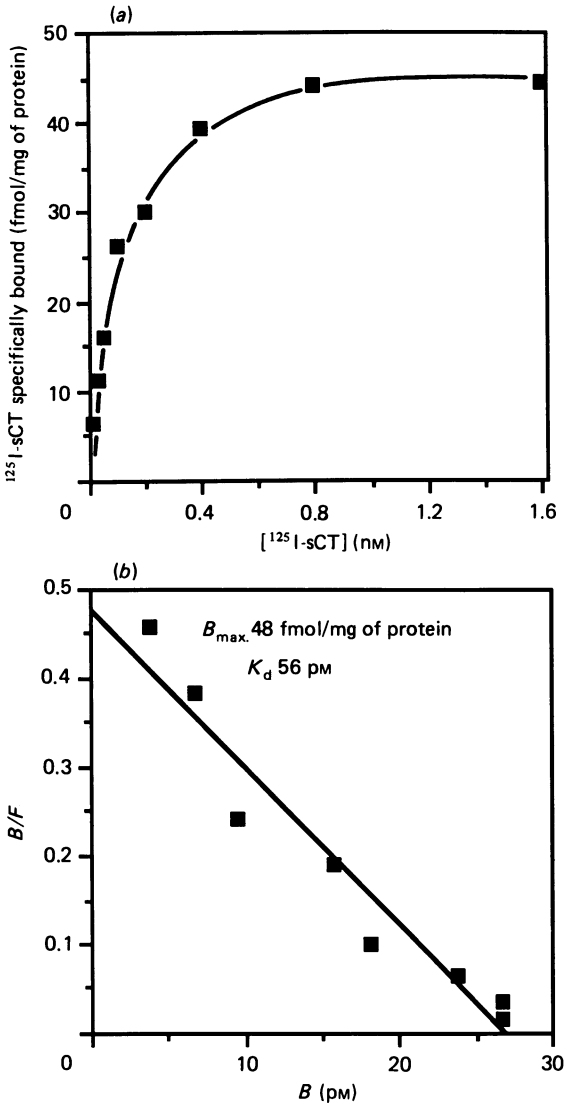

Fig. 2. Saturation analysis of ^{125}I-sCT binding to placental membranes

(*a*) Placental membranes (0.6 mg of protein/ml) were incubated with increasing concentrations of ^{125}I-sCT in the absence (total) or presence (nsb) of excess unlabelled sCT for 3 h at 22 °C. Bound and free radioactivity present in 100 μl aliquots were separated as described in the Materials and methods section. Points represent specific binding (total minus non-specific). (*b*) Scatchard analysis of specific binding data from (*a*).

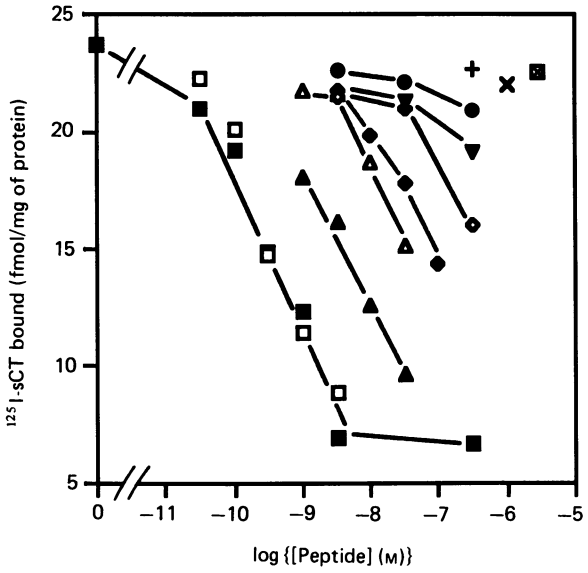

Fig. 3. Specificity of CT binding to placental membranes

Placental membranes were incubated with 0.1 nM-^{125}I-sCT with increasing concentrations of unlabelled sCT (■), [Asu$^{1-7}$]eCT (□), des-Leu^{16}-sCT (▲), pCT (△), hCT (◆), des-Phe^{16}-hCT (◇), [Gly^{8}]hCT (▼), hCGRP (●), ACTH-(1–22) (+), porcine insulin (×) or hPTH-(1–34) (⊠). Bound radioactivity was estimated as described in the Materials and methods section.

Time course of binding and dissociation

^{125}I-sCT binding was time-dependent, increasing to a maximum after 4 h at 22 °C (Fig. 1*a*). The addition of excess unlabelled sCT after 1 h incubation prevented further ^{125}I-sCT binding but resulted in minimal dissociation only (13 % loss of binding after 5 h), indicating that binding is essentially irreversible by ligand competition. The time course of association was similar at 0 °C with maximal binding occurring at 5 h. However, at 37 °C binding was maximal at 30 min and at 2 h and beyond showed progressive loss (Fig. 1*b*). This loss of binding was probably due to proteolysis of ^{125}I-sCT tracer at this higher temperature, since after 4 h only 66 % of radioactivity was precipitated by 5 % trichloroacetic acid (compared with 82 % of fresh tracer or tracer incubated with membranes for 4 h at 0 °C). Furthermore, at 37 °C nonspecific binding was very much greater, exceeding specific binding after 2 h. In view of these undesirable binding characteristics at 37 °C, subsequent studies were performed at 22 or 0 °C.

Saturation analysis

Saturation of binding to the CT receptor was demonstrated by incubating placental membranes with increasing concentrations of ^{125}I-sCT at 22 °C (Fig. 2*a*). Scatchard analysis [37] of the specific binding data (Fig. 2*b*) was linear, indicating a single class of binding sites with high affinity for sCT (K_d 56 pM, $B_{max.}$ 48 fmol/mg of protein). Another saturation analysis experiment performed at 22 °C gave similar results (K_d 85 pM, $B_{max.}$ 57 fmol/mg of protein), as did two competitive binding experiments performed at 22 °C (K_d 43 and 136 pM, $B_{max.}$ 87 and 38 fmol/mg of protein) (results not shown). Thus in four experiments performed at 22 °C, the mean (±S.E.M.) K_d was 80±21 pM and mean $B_{max.}$ 58±11 fmol/mg of protein. Saturation analysis experiments performed at 0 °C also gave comparable results (K_d 130 and 79 pM, $B_{max.}$ 23 and 25 fmol/mg of protein in two separate experiments) (results not shown).

Specificity of binding

To determine the specificity of CT binding to the receptor, competitive binding studies were performed with various CTs, CT analogues and other peptides. Species-specific CTs and analogues competed for ^{125}I-sCT binding (Fig. 3) with potencies (sCT = [Asu$^{1-7}$]eCT > des-Leu^{16}-sCT > pCT > hCT >

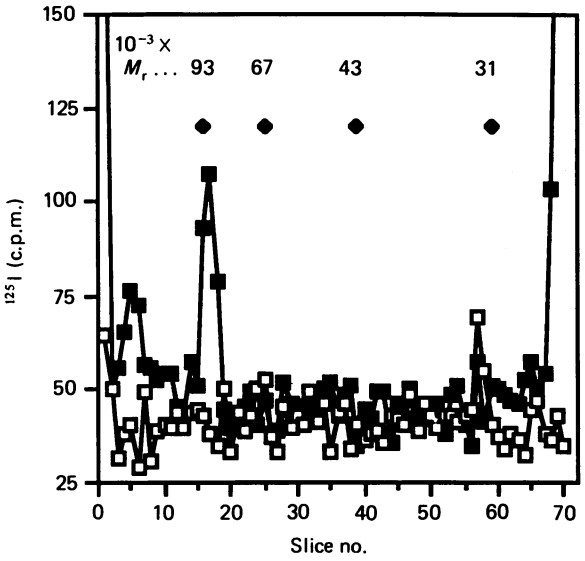

Fig. 4. Photoaffinity labelling of the placental CT receptor

Placental membranes were incubated with 0.2 nM-[Arg^{11,18}, 3-nitrophenylazido-Lys^{14}]sCT in the absence and presence of excess unlabelled sCT at 22 °C for 2 h in the dark. They were then washed, irradiated with a u.v. lamp for 15 min at 0 °C, solubilized in 0.1% Triton X-100 and subjected to SDS/PAGE under reducing conditions as described in the Materials and methods section. M_r standards used were phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase.

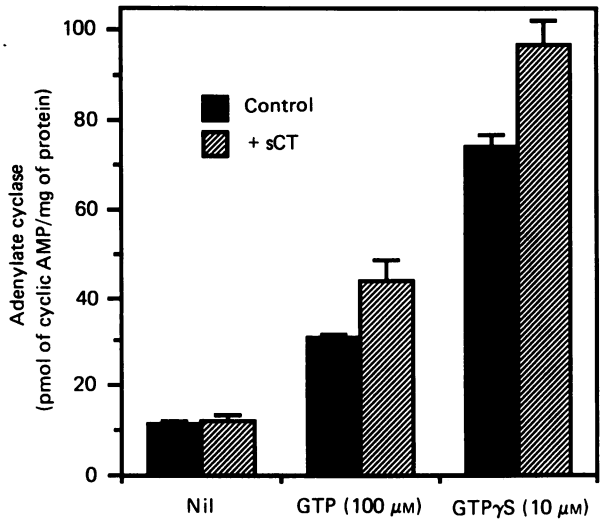

Fig. 5. Adenylate cyclase activity of placental homogenates

Homogenate was prepared as described in the Materials and methods section. Aliquots were incubated with and without 300 nM-sCT in the absence or presence of 100 μM-GTP or 10 μM-GTPγS. Adenylate cyclase activity was measured as described previously [36].

des-Phe^{16}-hCT > [Gly^{8}]hCT) proportional to their known biological potencies [38] and their abilities to compete for sCT binding to various cell lines [33,39–41]. hCGRP, which is a weak CT agonist [42–44], was a weak competitor at 3×10^{-7} M. The unrelated peptide hormones hPTH-(1–34), porcine insulin and ACTH-(1–24) did not compete at 3×10^{-6} M, 10^{-6} M and 3×10^{-7} M respectively.

Photoaffinity labelling

SDS/PAGE of extracts of placental membranes that had been photoaffinity labelled with ^{125}I-[Arg^{11,18}, 3-nitrophenylazide-Lys^{14}]sCT revealed a major peak of specifically bound radioactivity at an approximate M_r 85000–90000 (Fig. 4). It cannot be ruled out that this crosslinked component represents a subunit of a larger receptor complex. CT receptor components of M_r 85000–90000 have been identified on the human lung (BEN) and breast (T47D and MCF7) cancer cell lines using a photoactive sCT probe (sCT derivatized with 4-azido-2-nitroaniline at Gln^{14}) [45,46] and on isolated rat osteoclasts using chemical cross-linking of sCT with disuccinimidyl suberate [5]. A minor peak of higher-M_r labelling was also seen in the experiment depicted in Fig. 4. However, the precise M_r of this peak was not determinable on 10% acrylamide gels, and in other experiments employing 7.5–15% acrylamide gradient gels it was not seen.

Adenylate cyclase activity

Placental membranes which had been frozen and thawed and freshly prepared homogenate showed adenylate cyclase activity which was stimulated by GTP (10^{-7}–10^{-4} M), guanosine 5′-[βγ-imido]triphosphate (10^{-4} M), GTPγS (10^{-9}–10^{-5} M) and forskolin (10^{-6}–10^{-5} M). Treatment of either preparation with sCT alone (1–300 nM) had no significant effect in ten separate experiments. However, freshly prepared homogenates simultaneously treated with a high concentration of sCT and either GTP (10^{-4} M) or GTPγS (10^{-5} M) showed significant stimulation (Fig. 5).

DISCUSSION

These results demonstrate that the membrane fraction prepared from term human placenta contains CT binding sites that have similar specificity, kinetics and apparent M_r as CT receptors from other sources. Like the CT receptor of other target cells or tissues, the placental CT receptor has a high affinity for salmon CT, the relative binding potencies of CTs derived from other species are maintained and non-related peptides do not bind. Furthermore, the placental receptor displays poorly reversible binding, characteristic of CT binding in all tissues studied [7,10,20,39,40].

In the presence of poorly reversible binding, calculation of the K_d using Scatchard analysis will only give an approximate result, as we have previously discussed [5,33,40,41]. Even so, it is useful to compare our present results obtained from the placenta with those obtained from other tissues. Reported K_d values range three orders of magnitude from 12 pM in calvarial membranes [8] to 32 nM in testicular membranes [14], the latter probably being in the non-physiological range. However, the majority of reported K_d values have been in the range 110 pM–2 nM with sources including osteoclasts [5], renal membranes [8] and various cell lines [22,39,40]. Most tissues appear to possess a single class of CT receptors, although a second low-affinity site has been noted by some workers [8,11,15,47]. The reported maximum specific binding activities ($B_{max.}$) have ranged from 5 fmol/mg of protein for porcine lung membranes [13] to

190 fmol/mg of protein for rat hypothalamic homogenate [48]. Thus, the affinity of the placental CT receptor (mean K_d 80 ± 21 pM, $n = 4$) is comparable with that of other physiological targets such as osteoclasts [5]. Although the $B_{max.}$ (mean 58 ± 11 fmol/mg of protein, $n = 4$) is not as high as that obtained with some other tissues, this disadvantage is compensated for by the ready availability of the tissue.

In the tissues and cells examined previously, CT receptors have been shown to be linked to activation of adenylate cyclase, although in the rat hypothalamus CT causes inhibition of adenylate cyclase [12]. It is not possible to conclude from our results that CT receptors in the placenta are also coupled to adenylate cyclase, since stimulation of the enzyme was only seen with high hormone concentrations in the presence of added GTP or GTPγS. This could be a non-specific effect of the peptide, since dose-responsive effects were not demonstrated. However, the β-adrenergic agonist adrenaline (5×10^{-5} M) produced only a 1.5-fold stimulation of adenylate cyclase in placental membranes when used alone but a 2.5-fold stimulation when used together with GTP (10^{-5} M) [49]. The question of effects of CT on adenylate cyclase in the placenta requires further study.

The physiological significance of the presence of CT receptors in the placenta remains to be determined. It is well established that plasma calcium levels are higher in the foetus than in the mother [29,30,50] due to active transport of calcium by the placenta against a concentration gradient [51,52]. Furthermore, although there are conflicting data regarding the relative PTH levels in maternal and foetal blood, most investigators agree that CT levels are increased in the human foetus [29]. There is a linear relationship between foetal plasma calcium and foetal CT secretion rate [53], foetal CT levels are stimulated by calcium infusion [54–56], and CT infusion into the foetus lowers the plasma calcium level in most species tested [30]. These data suggest that CT has a role in the control of the foetal calcium level. It is possible that in the foetus, as in post-natal life, CT lowers the calcium level by inhibiting osteoclastic bone resorption. However, the finding of CT receptors in the placenta raises the possibility that CT may also act by direct inhibition of the placental calcium pump. Garel [30] has commented on this possibility as an explanation for the observation that maternal hypercalcaemia is not usually associated with foetal hypercalcaemia. There is evidence that maternal CT levels are also elevated [53,57,58].

Although under certain conditions CT clearly produces hypocalcaemia in mammals, its physiological role in mammalian calcium metabolism remains to be determined. Furthermore, its physiological function(s) in more primitive vertebrates such as Aves and Teleosts, which possess more potent CTs, is even more uncertain. The identification of CT receptors in the placenta highlights the need for further investigation of the role of CT in foeto-maternal physiology.

This work was supported by the National Health and Medical Research Council of Australia. T. E. is a Queen Elizabeth II Fellow.

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Received 27 July 1987/1 October 1987; accepted 16 November 1987