Adrenomedullin and CGRP interact with endogenous calcitonin-receptor-like receptor in endothelial cells and induce its desensitisation by different mechanisms

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Summary
Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) are related peptides with distinct pharmacological profiles. Calcitonin-receptor-like receptor (CRLR, now known as CL) can function as either an AM receptor or a CGRP receptor, when cotransfected with receptor-activity-modifying proteins (RAMPs) that define ligand-binding specificity. The aim of the present study was to determine the role of endogenously expressed CL (EndoCL) in generating endogenous AM and CGRP receptors. We raised anti-human CL antibody and identified microvascular endothelial cells (MVECs) as a major CL-expressing cell type in tissues by immunohistochemistry. Cultured MVECs continue to express EndoCL as well as fully active endogenous AM- and CGRP-sensitive receptors in vitro, as demonstrated by the ability of both peptides to induce migration and Akt phosphorylation. We therefore tested the hypothesis that endothelial EndoCL can interact with both AM and CGRP by examining receptor internalisation and desensitisation (loss of the ability to induce Akt phosphorylation). We found that agonist-mediated internalisation of EndoCL occurs in response to AM but not CGRP in MVECs. However, AM-induced EndoCL internalisation was blocked by antagonists of both AM and CGRP receptors: AM22-52 and CGRP 8-37, respectively. Furthermore, AM-induced EndoCL internalisation resulted in desensitisation not only of AM but also of CGRP receptors. Finally, CGRP also induced desensitisation of both endogenous AM and CGRP receptors, but did not mediate EndoCL internalisation despite interaction with this receptor. Thus, EndoCL interacts with both AM and CGRP, and simultaneously acts as a receptor for both peptides (i.e acting as an endogenous AM/CGRP receptor) in endothelial cells. Interaction with either ligand is sufficient to induce EndoCL desensitisation to both AM and CGRP, but differential mechanisms are involved since only AM induces EndoCL internalisation. These novel findings regarding regulation of EndoCL function in endothelial cells are likely to be of importance in conditions where AM or CGRP levels are elevated, such as cardiovascular disease, diabetes and inflammation.

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Key words: Adrenomedullin, CGRP, CRLR, Endogenous CL, Endothelial cell

Introduction
The calcitonin family of peptide hormones comprises six known members [calcitonin, amylin, two calcitonin-gene-related peptides (CGRP-α and CGRP-β), adrenomedullin (AM) and the recently discovered intermedin], which are involved in cellular proliferation, survival and migration (supplementary material, Fig. S1) (Poyner et al., 2002; Roh et al., 2004). AM and CGRP play significant roles in vascular biology (for reviews, see Hinson et al., 2000; Brain and Grant, 2004).

Circulating AM levels are upregulated in cardiovascular disease and sepsis (reviewed by Hinson et al., 2000); and local concentrations are elevated in some tumours (Satoh et al., 1995). Accumulating experimental evidence from animal models suggests a significant role for AM in these pathologies since it protects against hypertension and ischaemia- or injury-induced vascular remodelling, regulates blood vessel permeability in response to inflammatory mediators, and promotes tumour angiogenesis (Shindo et al., 2000; Matsui et al., 2004; Kawai et al., 2004; Brell et al., 2005; Oehler et al., 2002; Martinez et al., 2002). Similar to AM, CGRP is a significant player in maintaining vascular function, in addition to its known role as a neuropeptide, and has been implicated...
in pathophysiology of subarachnoid haemorrhage, migraine and coronary artery disease (reviewed by Brain and Cambridge, 1996). However, despite the proposed role for AM and CGRP in various pathologies, the therapeutic potential of regulating their action in humans remains unclear. This is a result of the limited information about the distribution of endogenous AM and CGRP receptors, as well as mechanisms regulating their function (Hay et al., 2004; Kuwasako et al., 2004).

Effects of AM and CGRP are mediated through heterodimeric receptors composed of calcitonin-receptor-like receptor (CRLR, now known as CL) and one of the three receptor-activity-modifying proteins (RAMPs), as demonstrated in receptor system reconstitution models (McLatching et al., 1998; Poyner et al., 2002). CL belongs to the family B of seven-transmembrane (7TM) G-protein-coupled receptors (GPCRs). The RAMP family comprises three members (RAMP1, RAMP2 and RAMP3) that share less than 30% sequence identity but a common topological organisation (reviewed by Sexton et al., 2001). RAMPs are essential for terminal glycosylation, cell-surface targeting and ligand-binding selectivity of CL (McLatching et al., 1998). The glycosylation state of the CL receptor is crucial to its properties. Only mature, fully glycosylated CL species are expressed at the cell surface and are selectively recognised by both AM and CGRP (Hilairet et al., 2001a). CL-RAMP heterodimer formation at the cell surface defines the ligand-binding selectivity of the CL glycoprotein (McLatching et al., 1998). RAMP1 promotes the expression of CGRP receptor, whereas co-expression of RAMP2 or RAMP3 with CL leads to the formation of AM receptors, termed AM1 and AM2, respectively (McLatching et al., 1998; Poyner et al., 2002).

On the basis of CL-RAMP cotransfectant models as reported above, it has been proposed that the known effects of AM and CGRP on vascular cells are mediated through pharmacologically distinct receptors that also mediate the actions of the endogenous CL (EndoCL) receptor in a similar manner (Kamitani et al., 1999; Kim et al., 2003). However, no interaction of either peptide with EndoCL in vascular cells has yet been demonstrated. Furthermore, whereas pharmacological studies suggest the heterogeneity among endogenous AM and CGRP receptors, their nature, and a role for CL in particular, remains unclear (reviewed by Sexton et al., 2001) (Poyner et al., 2002; Kuwasako et al., 2004; Hay et al., 2004). For example, the CL-RAMP1 heterodimer matches the pharmacology of the CGRP1 receptor in cell lines and tissues, and is mainly characterised by high affinity for GPR antagonists such as CGPRP, and IBN4096BS (Poyner et al., 2002). However, in some tissues, another CGRP-responsive receptor is present, the CGRP2 receptor, which has a low affinity for GPR antagonists. There is no simple molecular correlate for this receptor, but it has been suggested that the phenotype might be a result of expression of complexes such as CL-RAMP3 (AM2 receptor), since it has appreciable affinity to CGPRP (Hay et al., 2004).

The lack of consistent data using ideal reconstitution systems [i.e. equivalent species of RAMPs and CL proteins or unmodified (untagged) cDNA constructs] makes it difficult to judge the significance of pharmacological studies on transfected receptor heterodimers in relation to the endogenous receptor system (Sexton et al., 2001; Hay et al., 2004; Hay et al., 2005). Cellular components other than just CL and RAMPs could also be important for the expressed phenotype and the properties of CL-associated receptor subtypes, such as the magnitude of agonist-mediated responses and mechanisms of internalisation and desensitisation (for reviews, see Sexton et al., 2001; Hay et al., 2003) (Evans et al., 2000; Bomberger et al., 2005). Furthermore, little is known about the distribution of CL and RAMPs in tissues. In view of the above, and since the role for CL-RAMP heterodimers in generating functional endogenous AM and CGRP receptor subtypes has yet to be demonstrated, we have searched for a model to study EndoCL.

We initially investigated the distribution of CL in human tissues, and then analysed its properties (glycosylation state, subcellular localisation and function) both in normal conditions and when agonist levels are elevated) specifically in microvascular endothelium, as this is a major cell type exhibiting endogenous expression of the receptor in vivo. Here, we demonstrate that the endothelial EndoCL can interact with either AM or CGRP. We show that these interactions result in desensitisation of both endogenous AM and CGRP receptors, irrespective of which agonist was used for initial stimulation, and that this occurs through different mechanisms, since EndoCL internalisation was induced by AM but not by CGRP. We conclude that AM- or CGRP-induced desensitisation of EndoCL, as a key endothelial AM/CGRP receptor, might have adverse effects on endothelial cell biology in diseases associated with upregulated levels of either of these two ligands, such as cardiovascular disease, neoplasia and inflammation.

**Results**

GPCR CL is predominantly expressed by microvascular endothelium in vivo and in vitro

To examine CL distribution in human tissues, we have raised an anti-human CL antibody and characterised its specificity using transient transfection of human embryonic kidney (HEK293T) cells expressing functional human (h)CL-RAMP1 and hCL-RAMP2 receptor heterodimers and various tissues (Fig. 1). Polyclonal anti-hCL antibody LN-1436 specifically recognised both transiently expressed and endogenously expressed hCL receptor (Fig. 1). HEK293T cells transiently expressing hCL produced a distinct band of ~45 kDa (Fig. 1A). Signal of similar size was observed in the majority of tissue lysates (Fig. 1B). Co-expression of hCL with RAMP1 or RAMP2 in HEK293T caused the ~45 kDa band to disappear and a diffuse band of about 50-55 kDa to appear (Fig. 1A). This suggests that antibody LN-1436 can also recognise the terminally glycosylated form of the CL receptor (Hilairet et al., 2001a). This band was also present in tissues lysates (Fig. 1B).

We then used endoglycosidases F and H to differentiate between N-linked glycoproteins to confirm the nature of CL species that were detected by the antibody LN-1436 (Fig. 1). When the 45 kDa band (representing hCL expressed in the absence of RAMPs; supplementary material, Fig. S2, lane 1) was treated with endoglycosidase F, a single immunoreactive species of 37 kDa was seen after SDS-PAGE (supplementary material, Fig. S2, lane 2). Thus, the ~45 kDa form is a glycoprotein (supplementary material, Fig. S2, lane 1). The ~55 kDa form (derived from co-expression of hCL with RAMPs; supplementary material, Fig. S2, lanes 4, 7) was also reduced to a 37 kDa form by treatment with endoglycosidase F, demonstrating that the additional mass units represent carbohydrate residues (supplementary material, Fig. S2, lanes
The ~55 kDa form is resistant to endoglycosidase H, indicating that hCL has been terminally glycosylated, an event normally associated with transit through the Golgi complex and the production of mature glycoproteins (supplementary material, Fig. S2, lanes 6-9). The ~45 kDa form is sensitive to endoglycosidase H, indicating that it has not been terminally glycosylated. Thus, the antibody LN-1436 specifically recognises unglycosylated, core-glycosylated and mature transiently expressed and endogenously produced hCL.

We then used tissue microarray (TMA) technology (Kononen et al., 1998; Fox et al., 2004) to study hCL expression and localisation in tissues. The receptor is widely expressed in normal human tissues, including endometrium (Fig. 2A), myometrium (Fig. 2B), endometrial adenocarcinoma (Fig. 2C), corpus luteum (Fig. 2D), cervix (Fig. 2E,F), kidney, lung and others (supplementary material, Fig. S3A-D). In the majority, it is localised in microvascular endothelium (Fig. 2; supplementary material, Fig. S3), as further demonstrated by double immunofluorescence on frozen sections (supplementary material, Fig. S4). Furthermore, in some tissues, expression of hCL is not exclusive to endothelium in humans. Thus, hCL immunoreactivity was found in epithelium in cervix (Fig. 2F), kidney, adrenal gland and in some other organs (supplementary material, Fig. S3A-D, and summarised in supplementary material, Table S1). Our data support findings by others (Hagner et al., 2002), expanding the range of human organs investigated. Our data also demonstrate the heterogeneity of CL expression in vascular beds in various organs, since standardised analysis using our anti-hCL antibody and TMA was performed (summarised in supplementary material, Table S1).

We then asked whether microvascular endothelial cells (MVECs) isolated from various human organs (skin, lung, endometrium and myometrium) maintain expression of glycosylated EndoCL in vitro (i.e. reflecting RAMP co-expression and the functional state of the receptor). We also analysed its expression in other cell types from the non-endothelial cell lineage (epithelial, and vascular and non-vascular smooth muscle cells) in vitro. Highly endothelial-specific expression of hCL was observed in vitro (supplementary material, Fig. S5A). All MVECs studied expressed core-glycosylated and mature EndoCL, although the amount varied (supplementary material, Fig. S5B).

Correlations with the presence of the terminally glycosylated form of EndoCL

Correlation with RAMP mRNA expression

The presence of the terminally glycosylated form of EndoCL in MVECs directly suggests the presence of translated endogenous RAMP proteins, since CL could only be glycosylated and transported to the cell surface in the presence of these accessory proteins (McLatchie et al., 1998). We investigated which endogenous RAMPs are co-expressed with EndoCL in microvascular endothelium. Unfortunately, no anti-human RAMP antibodies that reliably distinguish RAMP isoforms (1, 2 and 3) or demonstrate expression of endogenous monomers of the expected molecular weight (which should be observed in the case of a heterodimeric CL-RAMP receptor) are available. Therefore, we studied the mRNA expression of RAMPs. We demonstrated that the presence of the terminally glycosylated form of EndoCL in all MVECs studied correlates with the expression of RAMP2 and RAMP3 mRNAs only (supplementary material, Fig. S6). None of the MVECs studied expressed RAMP1 mRNA (supplementary material, Fig. S6). Since all studied MVECs demonstrated expression of mature glycosylated EndoCL (supplementary material, Fig. S5) and a similar pattern of RAMP expression (supplementary material, Fig. S6), we focused our studies of the subcellular localisation and trafficking of EndoCL on human dermal MVECs (hDMVECs) (Fig. 3).
Desensitisation of endogenous endothelial CL

Correlation with cell-surface expression

The presence of the terminally glycosylated form of EndoCL suggests cell-surface expression of this receptor in hDMVECs. We analysed subcellular localisation of EndoCL in hDMVECs by immunofluorescence. Predominant surface staining was demonstrated by co-expression with the plasma membrane marker CD31 (Fig. 4A). Individual hDMVECs also displayed a punctuated staining pattern widely distributed in the cytoplasm as well as concentrated in a perinuclear region (Fig. 4B-D). To identify the intracellular compartments of accumulation of the endogenous receptor, immunofluorescence for markers of cellular structures and different organelles in the endocytotic pathway was performed. Endogenous hCL protein did not show significant overlap with the Golgi complex marker GM130 (Fig. 4B); but colocalised with calnexin, the endoplasmic reticulum marker (Fig. 4C). Intracellular EndoCL and mannose-6-phosphate receptor (M6PR), the late endosome marker, showed very similar distribution patterns with significant overlap of both diffuse cytosolic and perinuclear vesicles (supplementary material, Fig. S7E), although a few hCL-containing vesicles that lacked M6PR were also observed. Similarly, endogenous hCL was colocalised with lysosomes (Fig. 4D).

Fig. 2. Localisation of EndoCL in human tissues. Localisation of hCL was assessed by (A-F) immunohistochemistry on paraffin sections from TMAs using primary LN-1436 and secondary alkaline phosphatase-conjugated antibodies, and detected with Vector Red (red colour). Cell nuclei were counterstained with haematoxylin (blue colour). Note the predominant CL expression in microvascular endothelium in (A) endometrium, (B) myometrium, (C) adenocarcinoma, (D) corpus luteum, and (E and F) cervical stroma (arrows); and in (F) cervical epithelium (arrowheads). (For hCL expression in other tissues, see supplementary material, Figs S3 and S4.)

Fig. 3. Expression of endogenous hCL species and RAMP mRNA in endothelial cells. (A) Expression of endogenous hCL species was analysed by immunoblotting. hDMVEC lysates were treated with endoglycosidase F (F, lane 2), endoglycosidase H (H, lane 3) or vehicle (–, lane 1) before SDS-PAGE under reducing conditions and immunoblotting with polyclonal anti-hCL antibody LN-1436. Arrowheads, deglycosylated (~37 kDa); open diamonds, core-glycosylated (~45 kDa); black diamonds, mature fully glycosylated (~55 kDa) forms of the receptor. The ~55 kDa hCL species are reduced to a ~37 kDa hCL band after endoglycosidase F treatment, but are resistant to endoglycosidase H. For loading controls, the membrane was reprobed with an antibody against β-actin. The immunoblot is representative of two independent experiments. (B) Expression of CL and RAMP mRNAs in primary hDMVECs (lane 1) was analysed by RT-PCR. The set of primers for detection of β-actin was used as a loading control. RNA sample from kidney (lane 2) served as a positive control. Numbers to the right indicate PCR fragment size. (For details of a full RT-PCR screen of all endothelial cell lines used in the present study, see supplementary material, Fig. S5.)
By contrast, the receptor did not show marked overlap with early sorting endosomes, indicated by EEA1 (supplementary material, Fig. S7D). Thus, although EndoCL was predominantly localised at the cell surface, a significant portion accumulated in recycling endosomes, lysosomes and endoplasmic reticulum (Fig. 4; supplementary material, Fig. S7). This suggests that endogenous endothelial-cell-expressed hCL might cycle constitutively between the cell surface and recycling endosomes. EndoCL expression was found at apical, basal and lateral cell surfaces in hDMVECs by confocal microscopy (Fig. 4E), demonstrating a lack of preferential localisation at the cell surface.

Correlation with the presence of both functional endogenous AM and CGRP receptors
We then investigated whether the expression of mature glycosylated EndoCL correlates with the presence of functional endogenous AM or CGRP receptors in microvascular endothelium. The functional state of both receptor subtypes was investigated using cAMP, Akt phosphorylation and migration assays. We found that both endogenous AM and CGRP receptors are fully active in hDMVECs in vitro, as demonstrated by the comparable magnitude of increase in cAMP production (supplementary material, Fig. S8) and migration rates (Fig. 5; supplementary material, Fig. S9) in response to both ligands, and the induction of Akt phosphorylation as described in more detail below. cAMP accumulation and migration were stimulated by both peptides (10 nM to 1 μM) (Fig. 5; supplementary material, Figs S8, S9). Akt phosphorylation was also induced by both AM and CGRP as described in more detail below. Similar to hDMVECs, fully active endogenous AM and CGRP receptors were found in human myometrial MVECs (hMMVECs) in vitro, as demonstrated by the comparable magnitude of agonist-mediated migration (data not shown).

AM and CGRP interact with endothelial EndoCL and induce its desensitisation by different mechanisms
The presence of fully active receptors for both AM and CGRP suggest that endogenous hDMVEC-expressed CL might interact with both peptides. We tested this hypothesis by investigating the dynamics of EndoCL internalisation in hDMVECs in response to AM or CGRP (10 nM to 1 μM).

Internalisation of EndoCL
Agonist-mediated internalisation of endothelial cell-surface-expressed endogenous receptor was observed in response to AM but not CGRP (Fig. 6). Time-course studies demonstrated the existence of an early (within 5-15 minutes) and ligand-specific regulation of EndoCL internalisation by AM. A significant
proportion of EndoCL was initially (15-30 minutes) targeted to early sorting endosomes and then to lysosomes upon exposure to the 10 nM to 1 μM ligand (Fig. 6). Internalisation of the receptor occurred in all hCL-expressing cells (supplementary material, Fig. S10). Recycling of the internalised EndoCL in MVECs was not efficient, with a significant proportion of endocytosed hCL being targeted to the degradative pathway through lysosomes. The receptor reappeared on the cell surface only two hours after the removal of agonist (Fig. 6). By contrast, CGRP (10 nM to 1 μM) failed to demonstrate any changes in subcellular localisation of endogenous receptor (Fig. 6). These data suggest that EndoCL interacts with AM and generates endogenous AM receptor only. A similar specificity of EndoCL internalisation in response to AM but not CGRP was observed in hMMVECs (data not shown).

Kuwasaki et al. reported ligand-specific regulation of green fluorescent protein (GFP)-tagged CL (CL-GFP) internalisation by RAMPs (Kuwasaki et al., 2000). Thus, agonist-mediated...
internalisation of CL-GFP was observed in RAMP1 cotransfectants (by CGRP or AM), RAMP2 cotransfectants (AM only) and RAMP3-cotransfectants (AM only), which occurred with similar kinetics. These findings suggest that, in our study, endothelial EndoCL exists as a heterodimer with RAMP2 (AM1 receptor) or RAMP3 (AM2 receptor) (Fig. 6). This is supported by our findings on predominant expression of RAMP2 and RAMP3 but not RAMP1 mRNA in these cells (Fig. 3). However, in the same study, Kuwasako et al. reported that only the CL-RAMP1 complex shows similar functional responses to stimulation by both AM and CGRP (Kuwasako et al., 2000). Furthermore, other groups reported that transfected CL-RAMP3 complex (AM2 receptor) has appreciable affinity to CGRP (Poyner et al., 2002). Thus, it could not be excluded that EndoCL also generates an endogenous CGRP receptor (either CL-RAMP1 or CL-RAMP3 complex) in endothelial cells. In this case, the contribution of endogenous RAMP1 to the expression of EndoCL-associated CGRP receptor also could not be excluded because mRNA levels do not always reflect the protein levels and because the presence of the efficient RAMP1 translational machinery in endothelial cells has been previously demonstrated (Hay et al., 2003; Muff et al., 1998).

To test the hypothesis that EndoCL also generates an endogenous CGRP receptor, we investigated the dynamics of endogenous AM and Cgrp receptor desensitisation, and the effect of AM and Cgrp receptor antagonists on EndoCL internalisation. Agonist-promoted internalisation of GPCRs is often related to the loss of receptor activity (Koenig et al., 1997; Krupnick and Benovic, 1998). Since EndoCL is internalised after exposure to AM, we tested whether only endogenous AM or both endogenous AM and Cgrp receptors lose their activity (desensitise) under these conditions. Similarly, we tested the activity of both receptor subtypes after stimulation with Cgrp (when EndoCL is not internalised).

Desensitisation of endogenous AM and Cgrp receptors

Activation of Akt in endothelial cells plays an important role in AM- and vascular endothelial growth factor (VEGF)-induced angiogenesis (Ilan et al., 1998; Kim et al., 2003). We used an Akt phosphorylation assay to determine Akt activity and to investigate the dynamics of desensitisation of endogenous AM and Cgrp receptors.

Both AM (Fig. 7, lanes 2-4) and Cgrp (Fig. 7, lanes 11-13) (100 nM) increased Akt phosphorylation at Ser473 as early as 10 and 15 minutes, respectively, compared with the control (Fig. 7, lane 1), and to a similar degree to VEGF (Fig. 7, lane 10). Desensitisation assays revealed rapid (within 15 minutes) and stable desensitisation of endogenous endothelial AM and Cgrp receptors. Re-challenge with either agonist (AM, Fig. 7, lanes 5-8; or Cgrp, Fig. 7, lanes 14-17) after 30 minutes (with ligand depleted from the medium) demonstrated a lack of repeated Akt phosphorylation, and therefore suggests a sustained receptor desensitisation. Furthermore, reciprocal re-challenge of receptors (AM receptor with Cgrp, Fig. 7, lane 9; and Cgrp receptor with AM, Fig. 7, lane 18) revealed the loss of endogenous receptor activity.

Both AM and Cgrp interact with endothelial EndoCL

Since both endogenous AM and Cgrp receptors desensitise in response to either agonist, this suggests that both AM and Cgrp interact with the same receptor in endothelial cells. That this receptor is EndoCL was further supported by the use of AM22-52 and Cgrp8-37, which are putative antagonists of AM and Cgrp receptors, respectively (Poyner et al., 2002). Both truncated peptides blocked AM-induced internalisation of EndoCL in hDMVECs (Fig. 8). AM22-52 completely abolished, whereas Cgrp8-37 attenuated, internalisation of the receptor, supporting the view that both AM and Cgrp interact with endothelial EndoCL.

Fig. 7. Desensitisation of endogenous AM and Cgrp receptors in endothelial cells. The dynamics of desensitisation of endogenous AM and Cgrp receptors in hDMVECs was investigated using an Akt phosphorylation (Akt-P) assay to determine Akt activity. In control groups, cells were exposed to 100 nM AM (AM, lanes 2-4) or Cgrp (Cgrp, lanes 11-13) for indicated times. In pretreatment groups (Pretreatment with AM or Pretreatment with Cgrp; –/+ indicate if pretreatment was performed), cells were exposed to 100 nM AM (lanes 5-9) or Cgrp (lanes 14-18) for 15 minutes, and then to a ligand-depleted medium for a further 30 minutes. In pretreatment groups, cells were then re-challenged with either the same agonist (100 nM) (lanes 5-8 for AM, and lanes 14-17 for, respectively) or reciprocal agonists (100 nM) (Cgrp for AM-pretreated cells, lane 9; and AM for Cgrp-pretreated cells, lane 18) to examine receptor desensitisation. VEGF-treated cells (10 ng/ml for 10 minutes; lane 10) were used as a positive control.
Discussion

In the present study, we have described specific properties of the endogenous GPCR CL (EndoCL) expressed in human endothelium. We have shown its ability to interact with both AM and CGRP, and we have shown that interaction with either ligand is sufficient to induce receptor desensitisation to both AM and CGRP. Moreover, two different mechanisms are involved, and they seem to be equally important in regulating the function of endothelial EndoCL.

Endothelial cells present a unique model for studying EndoCL without the need to create ideal reconstitution systems, since these cells ubiquitously express mature glycoprotein (RAMP-associated, functional receptor) in vivo and in vitro, as well as functional endogenous AM and CGRP receptors. It has been hypothesised that the regulation of a cellular CGRP to AM responsiveness might be mediated by changes in RAMP expression, which would effectively desensitise a cell to CGRP and sensitise it to AM or vice versa (reviewed by Sexton, 2001). Recent studies have demonstrated that RAMP mRNA expression is dynamically regulated in several models of pathophysiological challenge and/or drug treatment (reviewed by Sexton, 2001); however, whether this leads to a switch between receptor subtypes was not investigated. Our study suggests an alternative mechanism of EndoCL regulation.

Our findings suggest that the mechanisms regulating endothelial EndoCL function are based primarily on its ability to interact with both agonists (AM and CGRP), as well as differential consequences of such interactions, and not on a switch between AM and CGRP receptor subtypes (Fig. 9). Our findings that CL can generate AM or CGRP receptors parallel results from other studies (McLatchie et al., 1998), but the finding that endothelial EndoCL simultaneously acts as a receptor for both peptides (i.e. plays a role as an endogenous AM/CGRP receptor) is new (Fig. 9). Furthermore, our study not only supports previous reports suggesting that both AM and CGRP interact with the same receptor in endothelial cells (Shichiri et al., 1999), but also demonstrates for the first time that this receptor is EndoCL.

It remains to be investigated which RAMP forms a heterodimer with endothelial EndoCL, because its properties do not entirely match those observed for CL-RAMP cotransfectants (McLatchie et al., 1998; Kuwasako et al., 2000). For example, RAMP mRNA expression profile (lack of RAMP1 mRNA) and internalisation studies suggest that endothelial EndoCL should be exhibiting AM1 and/or AM2 receptor phenotype. But because EndoCL also interacts with CGRP, CL-RAMP3 is likely to be a predominant receptor complex in endothelial cells, since appreciable affinity to CGRP has been demonstrated for this heterodimer in transfected cell models (Hay et al., 2005). Given the data suggesting a potential role for G-protein coupling in the expression of RAMP-induced phenotype (reviewed by Sexton et al., 2001), it is possible that other components of the cellular background could also be important in defining the observed phenotype and properties of endothelial EndoCL. This is because the CL-RAMP3 complex, in contrast to CL-RAMP1 and to endothelial EndoCL, does not exhibit similar functional responses to AM and CGRP peptides (Kuwasko et al., 2000).

Nevertheless, irrespective of the RAMP isoform involved, fully glycosylated EndoCL glycoprotein (functional receptor) is produced in endothelial cells, as demonstrated using anti-hCL antibody (summarised in Fig. 9). Interaction with either ligand is sufficient to induce its desensitisation to both AM and CGRP, but different mechanisms are involved. Thus, AM-induced complete desensitisation of endogenous AM/CGRP receptors coincides with EndoCL internalisation and its targeting to the degradation pathway with subsequent...
reappearance on the cell surface only 2 hours after an initial challenge. By contrast, CGRP-induced desensitisation of endogenous AM/CGRP receptors is associated with retention of EndoCL at the cell surface.

To our knowledge, there are no data on endogenous AM or CGRP receptor desensitisation in endothelial cells or underlying mechanisms. Agonist-promoted internalisation is common to a large number of GPCRs and is often related to the loss of receptor activity (Koenig et al., 1997; Krupnick and Benovic, 1998). Thus, our findings that AM-induced EndoCL internalisation results in desensitisation of endogenous AM receptors parallel results from other studies, including those using CL-RAMP receptor system reconstitution models (Kuwashako et al., 2000; Hilairet et al., 2001b), but the finding that it also results in loss of cell sensitivity to CGRP is new. It is notable that CGRP is also able to interact with endothelial EndoCL and to induce desensitisation of both endogenous CGRP and AM receptors, thus supporting the view that both peptides act through the same receptor. However, the mechanism of CGRP-induced desensitisation of EndoCL does not involve receptor internalisation and therefore requires further investigation. For example, it might involve GPCR kinases (GRK). Seven GRKs have been identified to date, and they play a pivotal role in desensitisation of GPCRs without loss of their cell-surface expression (Penn et al., 2000). Thus, a role for GRK6 in the regulation of CGRP-mediated desensitisation of porcine CL transiently expressed in HEK293T cells has been suggested (Aiyar et al., 2000). In endothelial cells, desensitisation of GPCRs might involve GRK2 (Liu et al., 2005). Thus, the activated endothelin receptor (ETB), a canonical GPCR, recruits GRK2 that binds directly to Akt and inhibits its phosphorylation in endothelial cells (Liu et al., 2005). We speculate that a similar mechanism could also be involved in CGRP-induced desensitisation (loss of ability to induce Akt phosphorylation) of endothelial
EndoCL. However, irrespective of the mechanism involved in EndoCL desensitisation, our findings suggest that endogenous endothelial AM and CGRP receptors might lose their activity through sustained desensitisation after interaction with either ligand, rather than switching between receptor subtypes/phenotypes, since EndoCL simultaneously acts as a receptor for both peptides.

Desensitisation of EndoCL as a key endothelial AM/CGRP receptor might have adverse effects on endothelial cell biology in pathologies where levels of either peptide are elevated, such as cardiovascular disease, neoplasia and inflammation (where AM levels are elevated); and subarachnoid haemorrhage and migraine (where CGRP levels are upregulated). This is because AM signalling is essential in endothelial cell biology, as demonstrated in AM-‘depleted’ or ‘deficient’ endothelium in vivo and in vitro (Shindo et al., 2001; Hippenstiel et al., 2002). Thus, lack of basal-cell-surface AM signalling through EndoCL as a key endothelial AM/CGRP receptor could contribute to changes in endothelial cell morphology and their detachment from the basal membrane in vitelline vessels found in AM-homozygote knockout mice (Shindo et al., 2001).

Blockade or insufficient AM cross-talk between endothelial cells through juxtacrine loops mediated by laterally expressed EndoCL might contribute to hyperpermeability of the endothelial monolayer in response to stress stimuli, such as polymorphonuclear-leukocyte-derived oxygen metabolites (Hippenstiel et al., 2002). Apically expressed EndoCL might mediate endothelium-dependent AM-induced vasodilatation (Hinson et al., 2000; Zhang and Hintze, 2001). Similarly, EndoCL could be important in mediating CGRP-mediated proliferation (Haegerstrand et al., 1990) and migration (present study) of endothelial cells. However, further studies are required to dissect these mechanisms in order to understand the role of GPCR CL in transduction of signals from various members of the calcitonin family of peptide hormones.

The unregulated desensitisation of endothelial GPCRs can contribute to the aetiology of GPCR-based vascular diseases, since these receptors play important roles in vascular biology in mediating signal transduction from survival and angiogenic stimuli (Liu et al., 2005; LeCouter et al., 2003; Nikitenko et al., 2003; Maguire and Davenport, 2005). For example, injury-induced alterations in GPCR-mediated signalling in sinusoidal endothelium result in endothelial dysfunction and leads to intrahepatic portal hypertension (Liu et al., 2005). In human heart failure, desensitisation of GPCR-mediated beta-adrenergic signal transduction has been reported to be one of the main pathophysiological alterations (Schotten et al., 2000). Additionally, hypertension is accompanied by increased levels of endothelin-1 (ET-1) and decreased arterial contraction in response to ET-1 owing to desensitisation of its cognate GPCR (ET\(_{\text{A}}\)) (Thakali et al., 2004). Similarly, altered signalling through AM and CGRP receptors has been observed in conditions where ligand levels are upregulated but the underlying mechanisms remain unclear. For example, in chronic heart failure, a cardiovascular disease where plasma levels of AM are raised, the potent and long-lasting vasodilatory effect of AM on skeletal muscle arteries is significantly attenuated, in part because of impaired production of nitric oxide (NO) in the forearm resistance vessels (Kato et al., 1996; Nakamura et al., 1997). Our findings suggest that these effects could be a result of the EndoCL internalisation and desensitisation in endothelial cells, since studies by other groups have demonstrated a role for AM in the regulation of blood pressure by stimulating NO production and have shown that the NO pathway is essential for AM-induced endothelium-dependent vasodilatation in particular (Shindo et al., 2001; Zhang and Hintze, 2001).

The precise mechanisms underlying the response of endogenous AM and CGRP receptors to constitutive/chronic agonist stimulation in conditions such as cardiovascular disease, neoplasia and inflammation are yet to be investigated. Detailed knowledge of mechanisms regulating the function (sensitisation/desensitisation) of EndoCL in endothelium might enable better understanding of alterations in vascular responses in these conditions. Therefore, the molecular mechanisms of trafficking, function and desensitisation of endogenous GPCR CL, as a key AM/CGRP receptor, require further investigation. Detailed knowledge of these mechanisms might provide insights for the development of novel compounds and antibodies that can modulate the activity of endogenous AM and CGRP receptors. Such drugs might have potential in cardiovascular disease and in cancer (Doods et al., 2000; Martinez et al., 2004).

In summary, our data demonstrate for the first time that the function of EndoCL is tightly regulated in endothelial cells. The finding that endogenous endothelial CL can interact with both AM and CGRP, as well as that either of these interactions can result in complete desensitisation of the receptor, suggests that the mechanisms regulating expression and function of this GPCR in endothelium have the potential to lead to translational advances in vascular therapeutic methods and techniques for conditions where levels of AM or CGRP are elevated.

Materials and Methods

Construction of human CL cDNA vector and expression of functional receptor

The full open reading frame (ORF) of the reported human (h)CL, modified to provide a consensus Kozak sequence (Aiyar et al., 1996), was cloned into pCNA3.1+ vector (Invitrogen) at its multicloning site, using RNA isolated from MVECs as a template (Nikitenko et al., 2003). 1 \( \mu \)g of total RNA was reverse-transcribed (RT) with an oligo (dT) adaptor primer to generate the first-strand DNA using the Reverse-iTcDNA synthesis Kit (Advanced Biotechnologies), as previously described (Nikitenko et al., 2001; Nikitenko et al., 2003). The full ORF was amplified using appropriate primers containing XhoI and KpnI restriction sites at the 5' and 3' ends, respectively. The 5' and 3' adaptor sequence ends of hCL were ligated into the XhoI and KpnI sites of pcDNA 3.1+ (Invitrogen Life Technologies) at its multicloning site. The fused product was termed hCLpcDNA and sequenced using an Applied Biosystems 377 Genetic analyser. Myc-RAMP1 and Myc-RAMP2 cloned into pcDNA3.1 were a gift from D. Poyner (Aston University, Birmingham, UK), and were originally prepared by Dr S. Foord and co-workers (GlassSmithKline, UK).

1 \( \mu \)g DNA per plasmid was transfected for each T 75 cm\(^2\) flask containing 50-60% confluent HEK293T cells. Cells were transfected with hCLpcDNA alone or cotransfected with RAMP1 and RAMP2 cDNAs. Cells were left for 24 hours before either plating into 96-well plates for intracellular cAMP assay (as described below) or transfers into 60% confluent HEK293T cells. Protein lysates from either plating into 96-well plates for intracellular cAMP assay (as described below, and to validate expression of functional specific AM and CGRP receptor subtypes upon hCL and RAMP cotransfection in HEK293T cells). Protein lysates from HEK293T transfectants expressing functional CL-RAMP heterodimers were used in the present study for immunoblotting analysis solely for the purpose of validation of specificity of our own polyclonal antibody (see below).

Antibody production and characterisation

Rabbit polyclonal antibody LN-1436 was raised against synthetic peptide corresponding to residues 427-481 (HDENVLLKPNLNY) at the extreme C-terminus of hCL protein (Accession numbers AAC41994 and AAA62158) (Sigma Genosys). The peptide antigen was selected based on high sequence specificity (CLASTP analysis; http://www.ncbi.nlm.nih.gov). The hCL peptide was conjugated to keyhole limpet haemocyanin using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride before being used for immunisation of New Zealand White rabbits. The titres of the resulting antisera were accessed by enzyme-
Human VEGF was from R&D Systems.

Peptides and growth factors
Synthetic human AM, α-CGRP, AM$_{22-25}$, CGRP$_{1-27}$ and amylin were from Bachem. Human VEGF was from R&D Systems.

Cell culture

Primary MVEC lines
Primary human dermal, lung, endometrial and myometrial MVECs and non-endothelial cell lines were obtained and cultured as previously described (Nikitenko et al., 2000; Nikitenko et al., 2003). Cells were seeded onto plastic culture dishes (Falcon; $\approx 5 \times 10^5$ cells/cm$^2$) precoated with Attachment Factor (TCS Cellworks) according to the manufacturer’s instructions, and supplemented with EGM-2MV Bullet Kit medium (Bio Whittaker). Cultures were incubated at 37°C in a 5% CO$_2$ humidified atmosphere and the medium was replaced every 1-2 days. Cells were passaged 1:3 at confluence by trypsin/EDTA (Sigma).

Non-endothelial cell lines
Several cell lines were chosen to serve as controls by representing cells from the non-endothelial cell lineage. These included epithelial, vascular and non-vascular smooth muscle cells. Primary myometrial smooth muscle cells were isolated by collagenase digestion from uteri obtained at hysterectomies in the Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital, Oxford. MDA 231 (a breast epithelial carcinoma cell line) and HEK293T (human renal epithelial cells) were obtained from the American Tissue Culture Collection (ATCC) (Tokesi et al., 1981; Wierland et al., 1996). Primary coronary artery vascular smooth muscle cells (VSMCs) were obtained from Cambrex and maintained according to the supplier’s protocols. All other non-endothelial cell lines were maintained in Dulbecco Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS), maintained at 37°C and 95% humidity, and passaged 1:3 at confluence.

Cell migration assays
Primary MVECs were used to study the effect of human AM, CGRP and amylin on migration. Human dermal MVECs were seeded into the upper chamber of Transwell inserts (BD Biosciences). Test peptides (human AM, CGRP and amylin) were added to the lower chamber. Full microvascular endothelial growth medium EGM-2MV and vascular endothelial growth factor were used as a positive control. After 24 hours incubation, the cells were labelled with a fluorescent dye (calcine AM; Molecular Probes). The number of cells migrated to the lower surface of the insert was determined by measurement of fluorescence of invaded cells in a fluorescence plate reader Elx 800 with bottom reading capabilities at excitation/emission wavelengths of 485/530 nm. Data were analysed with KC4v30 PowerReports Software and photographs taken with a Nikon CoolPix 990 camera. Only those labelled cells that have migrated through the pores of the FluoroBlok membrane can be detected.

Deglycosylation experiments
Glycosidases F and H were used to differentiate between N-linked glycoproteins (McLatchie et al., 1998). Cell lysates were treated with glycosidases F (which deglycosylates both the mature and core-glycosylated forms of the receptor) and H (which deglycosylates only the core-glycosylated form) according to the supplier’s protocols (Roche) before SDS-PAGE and immunoblotting with the antibody LN=1436.

SDS-PAGE and immunoblotting
Protein lysates from cell lines and tissues were subjected to SDS-PAGE and immunoblotting as previously described (Nikitenko et al., 2001). In brief, upon reaching confluence, cells were collected into the lysis buffer and homogenised. Cell lysates were subjected to SDS-PAGE and immunoblotting with primary anti-hCL polyclonal antibody LN 1436, followed by incubation with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Dako). The signal was developed using enhanced chemiluminescence ECL Plus (Amerham-Pharmacia) or Supersignal (Pierce & Warriner) systems. The membranes were then reprobed with anti-human β-actin antibody (Clone AC-15; Abcam) to monitor and confirm equal loading of total protein in samples. Anti-human CD31 antibody was used to demonstrate the purity of endothelial cell lines. The density of bands was analysed on an Alpha Imager TM 1220 Documentation & Analysis System v.5.5 using linked background subtraction.

Intracellular CAMP assays
For cAMP determinations, the transfected HEK293T cells were cultured for a further day, washed with PBS and preincubated in DMEM medium containing 300 μM IBMX (Sigma) for 30 minutes at 37°C. Forskolin (300 μM), CGRP or AM were added for a further 15 minutes.

MVECs at 3rd-5th passage were seeded into the 96-well microtitre plates (tissue-culture grade) precoated with Attachment Factor (TCS Cellworks) with cell concentrations of 8000 per well and grown for three days in EGM-2MV medium at 37°C in a 5% CO$_2$ humidified atmosphere and the medium was replaced daily. Cells were supplemented with medium containing 0.5% FCS (0.5%FCS-EGM-2) for 16 hours and then pretreated with the same medium containing phosphodiesterase type III inhibitor IBMX (300 μM Sigma) for 30 minutes at 37°C. The effect of forskolin (300 μM) or human AM, CGRP, amylin, VEGF (at a range of concentrations from 1 pM to 1 μM) (four wells for each dilution), or full medium (EGM-2-MV) on cAMP formation was examined. Plates were incubated for 15 minutes at 37°C.

After exposure to stimuli, and to terminate the reaction, HEK293T cells and MVECs were washed with ice-cold PBS. Intracellular CAMP measurement was performed using the non-acetylation EIA assay (Amersham Pharmacia Biotech) according to the manufacturer’s instructions and essentially as described previously (Nikitenko et al., 2000).

FACS analysis
Dermal MVECs were grown as described, collected by incubation with accutase (PAA Laboratories) for 10-20 minutes, fixed in 4% paraformaldehyde, then pelleted and washed three times in PGB Buffer (PBS, 20 mM glucose, 0.1% BSA). The cells were then resuspended in PGB Buffer (PBS, 20 mM glucose, 5% normal goat serum) and preincubated for 20 minutes, followed by the incubation for 25 minutes with the primary polyclonal anti-hCL LN-LN 1436 antibody diluted 1:1000 in PGN. Following three further washes in PGB Buffer, cells were incubated with the secondary antibody (goat anti-rabbit FITC-conjugated IgG; diluted 1:100) for 30 minutes in the dark. Finally, cells were washed in PGB Buffer and resuspended in PBS, 2% fetal calf serum, 0.1% sodium azide. For permeabilisation, cells were incubated with rotation in PBS, 0.1% BSA; 0.1% saponin for 20 minutes at 4°C, prior to labelling with primary antibodies. FACS analysis was performed on a Beckman Coulter EPICS Altra Elite (Coulter) using 488 nm line of the UV laser. Lasers were aligned using Flow Check Beads (Coulter) prior to experiment.

RNA isolation and RT-PCR
RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR) were performed as previously described (Nikitenko et al., 2001; Nikitenko et al., 2003). Amplifications were routinely performed using 25-30 cycles in the Perkin Elmer Gene Amp PCR System 2400 for β-actin control, AM, CL and RAMPs using primers designed for their specificity and spanning neighbouring exons to enable the detection of any genomic DNA contamination (supplementary material, Table S2).

Immunohistochemistry and immunofluorescence
Cultured MVECs and cryostat sections were prepared and processed for immunohistochemistry and immunofluorescence as previously described (Nikitenko et al., 2000; Nikitenko et al., 2001).

Cultured cells
MVECs were grown to confluence on attachment-factor-precoated 8-well Permanox slides (Nunc). Prior to immunohistochemistry, cultures were fixed in ice-cold acetone/methanol for 3-5 minutes and air dried for a minimum of 2 hours. Slides were stored at –20°C prior to staining. In parallel experiments, cells also were fixed in 4% paraformaldehyde, washed and stored in PBS at 4°C.

Cryostat sections
Endometrial tissues were snap frozen in liquid nitrogen and stored at –70°C. Frozen sections 8-10 μm were cut on a Leica Kryostat 1720 Digital cryostat, allowed to dry at room temperature and then fixed for 5 minutes in 4% ice-cold paraformaldehyde solution in PBS. Sections were then rinsed three times for 2 minutes in PBS, dehydrated sequentially in 70% and absolute ethanol and stored at 4°C in absolute ethanol (Nikitenko et al., 2001). Before immunohistochemistry, frozen sections were dried, washed for 5 minutes in distilled water and rinsed in Buffer 1 (100 mM Tris- HCl, 150 mM NaCl pH 7.5) for 5 minutes.

Multiple tissue microarray
Formalin-fixed, paraffin-embedded specimens (n=74) of 20 normal human tissues were selected from archival files of the Department of Cellular Pathology, John Radcliffe Hospital, University of Oxford, Oxford, UK. Multiple tissue microarrays (TMAs) were produced by acquiring cylindrical cores (1.0 mm diameter) for each specimen arrayed at high density into a recipient TMA block (Kononen et al., 1998). Consecutive sections were used for controls or for analysis with other antibodies as indicated below.
Antibodies
Identification of individual cell types and intracellular structures was performed according to the staining pattern with primary monoclonal and polyclonal antibodies as indicated below.

Immunocytochemistry and immunofluorescence
Immunocytochemistry and immunofluorescence were performed as previously described (Nikiteenko et al., 2000; Nikiteenko et al., 2001). For IHC detection in vivo on TMA, a two-stage anti-rabbit HRP with the EnvVision (Dako) system, which is not affected by the endogenous biotin that is present in some human tissues, was used. All immunocytochemistry or immunofluorescence reactions were followed by final washes and detection. When biotinylated secondary antibodies were used, final washes were followed by application of streptavidin-alkaline phosphatase (all from Dako). Secondary antibodies were Texas Red (TR) and fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse and horse anti-rabbit IgG, biotinylated horse anti-mouse and anti-goat antibodies (all Vector Labs), and HRP-conjugated swine anti-rabbit antibody (Dako).

Immunohistochemistry and immunofluorescence
Immunohistochemistry and immunofluorescence were performed as described (Koenig, J. A. and Edwardson, J. M. 1997). A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J. Biol. Chem. 271, 11325-11329.

References


Table S1. Tissue and cell types expressing hCL protein using a semi-quantification intensity scale*

<table>
<thead>
<tr>
<th>Organ</th>
<th>Endothelium</th>
<th>CL-IR</th>
<th>Other cell types</th>
<th>CL-IR</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>Endothelium</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>Endothelium</td>
<td>++++</td>
<td>Epithelium</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Myometrial smooth muscle</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>Endothelium</td>
<td>–/+</td>
<td>Pericytes</td>
<td>–/+</td>
<td></td>
</tr>
<tr>
<td>Gall bladder</td>
<td>Endothelium</td>
<td>++++</td>
<td>Smooth muscle cells</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mucous epithelium</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Endothelium</td>
<td>++++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil</td>
<td>Endothelium</td>
<td>+++</td>
<td>Squamous epithelium</td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymphoid cells</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>Endothelium</td>
<td>–/+ or +++</td>
<td>Epithelium-chief cells</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Epithelium-parietal cells</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>Endothelium</td>
<td>++++</td>
<td>Epithelium</td>
<td>–/+</td>
<td></td>
</tr>
<tr>
<td>Salivary gland</td>
<td>Endothelium</td>
<td>–/+ or ++</td>
<td>Duct epithelium</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Serous epithelium</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Parathyroid</td>
<td>Endothelium</td>
<td>–/+</td>
<td>Epithelium</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>Testis</td>
<td>Endothelium</td>
<td>+/-</td>
<td>Spermatogonia</td>
<td>++++</td>
<td>Strong NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Leydig cells</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Trachea</td>
<td>Endothelium</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Endothelium</td>
<td>++++</td>
<td>Epithelium</td>
<td>+++</td>
<td></td>
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<tr>
<td>Oesophagus</td>
<td>Endothelium</td>
<td>++</td>
<td>Vascular smooth muscle cells</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Epidermis</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>Epithelium</td>
<td>–/+</td>
<td>Vascular smooth muscle cells</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Endothelium</td>
<td>+</td>
<td>Hepatocytes</td>
<td>++++</td>
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<tr>
<td>Pancreas</td>
<td>Endothelium</td>
<td>+/-</td>
<td>Beta-cells</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glandular exocrine cell</td>
<td>+/-</td>
<td>NS</td>
</tr>
<tr>
<td>Placenta</td>
<td>Endothelium</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amnion</td>
<td>Endothelium</td>
<td>–</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*++++, very strong expression; ++++, strong expression; ++, medium expression; +, weak expression; –, no expression; N/A, not assessed; NS, nuclear staining; CL-IR, CL immunoreactivity.
Table S2. RT PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Primers (5'-3') Forward</th>
<th>Primers (5'-3') Reverse</th>
<th>Sequence</th>
<th>PCR fragment size</th>
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<tr>
<td>CL</td>
<td>U 17473</td>
<td>CTCCTCTACATTATCCATGG</td>
<td>CCTCCTCTGCAATTTCC</td>
<td>1338-1560</td>
<td>222</td>
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<td></td>
<td></td>
<td>exon 12</td>
<td>exon 13</td>
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<tr>
<td>RAMP1</td>
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<td>160-481</td>
<td>321</td>
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<td></td>
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<td>exon 2</td>
<td>exon 3</td>
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<td>RAMP2</td>
<td>NM 005854.1</td>
<td>AAAGGATTTGGTGCAGACTG</td>
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<td>308-635</td>
<td>327</td>
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<td>RAMP3</td>
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<td>118-519</td>
<td>401</td>
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<td>exon 2</td>
<td>exon 3</td>
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<tr>
<td>beta-Actin</td>
<td>NM 001101</td>
<td>ATCACCATTGGCAATGAGCG</td>
<td>TTGAAGGTTGTTCGGAT</td>
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<td>97</td>
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<td></td>
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<td>exon 4</td>
<td>exon 5</td>
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