CGRP, adrenomedullin and adrenomedullin 2 display endogenous GPCR agonist bias in primary human cardiovascular cells

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Agonist bias occurs when different ligands produce distinct signalling outputs when acting at the same receptor. However, its physiological relevance is not always clear. Using primary human cells and gene editing techniques, we demonstrate endogenous agonist bias with physiological consequences for the calcitonin receptor-like receptor, CLR. By switching the receptor-activity modifying protein (RAMP) associated with CLR we can "re-route" the physiological pathways activated by endogenous agonists calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and adrenomedullin 2 (AM2). AM2 promotes calcium-mediated nitric oxide signalling whereas CGRP and AM show pro-proliferative effects in cardiovascular cells, thus providing a rationale for the expression of the three peptides. CLR-based agonist bias occurs naturally in human cells and has a fundamental purpose for its existence. We anticipate this will be a starting point for more studies into RAMP function in native environments and their importance in endogenous GPCR signalling.

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protein-coupled receptors (GPCRs) form the largest protein family in the human genome. Approximately 30% of marketed drugs target these receptors and therefore understanding their signalling pathways is not simply an academic exercise. For many years it had been incorrectly assumed that agonist-occupied GPCRs signalled through a single pathway to elicit their response. However, there is now overwhelming evidence to suggest that many GPCRs exist in multiple receptor conformations and can elicit numerous functional responses, both G protein- and non-G protein-dependent. Furthermore, different agonists, acting at the same receptor have the potential to activate different signalling pathways to varying extents; a concept referred to as biased agonism or signalling bias. This can explain why there is apparent duplication amongst endogenous agonists, particularly for peptides. While the therapeutic promise of biased agonists is obvious, it allows design of ligands that actively engage with one beneficial signalling outcome while reducing the contribution from those that mediate more undesirable effects. For example, recent doubt has been cast on the validity of developing synthetic biased agonists against the µ-opioid receptor—a GPCR considered the trailblazer for therapeutic potential of biased agonism. Further investigations into the role of agonist bias and its physiological importance, particularly its relevance to endogenous agonists, are required to bridge the gap between heterologous studies and in-vivo investigations. While there are many well-studied GPCRs that exhibit signalling bias, including the aforementioned µ-opioid receptor, we have focused upon the calcitonin-like receptor (CLR) since it couples to multiple G proteins and β-arrestins. Importantly, when co-expressed with one of three receptor-activity modifying proteins (RAMPs), it can be activated by distinct endogenous agonists; calcitonin–gene related polypeptide (CGRP), adrenomedullin (AM) and adrenomedullin 2/intermedin (AM2). This makes it a good system to investigate the role of bias for endogenous ligands. CGRP, an abundant neuropeptide, is the most potent microvascular vasodilator known. While it is thought to be cardioprotective, it has also been implicated in diseases such as migraine; AM is released by the vascular endothelium and is a potent vasodilator that can modulate vascular tone, it is involved in angiogenesis, and is elevated in some cancers and heart failure. AM2 is also a vasodilator and highly expressed in the heart and vasculature. It can cause sympathetic activation, have antidiuretic effects, and is upregulated in cardiac hypertrophy and myocardial infarction.

Moleculely, CLR and its close relative, the calcitonin receptor, are classical class B1 GPCRs. CLR is pleiotropically coupled, predominately activating Gs, although there are reports of couplings to both Gs and Gq/12 families. These Ga subunits promote activation/inhibition of adenyl cyclase and phospholipase Cβ to generate intracellular second messengers including cAMP and mobilise intracellular Ca2+. Then activate their respective intracellular signalling cascades. Beyond the coupling to Gi/o and Gq/112,12,12, these Ga subunits can activate both G protein- and non-G protein-dependent. Furthermore, AM1 receptor, we demonstrate that biased agonism is present and has a fundamental role in the function of peptide hormones acting on primary human cells. Moreover, through deletion of the endogenous RAMP2 and replacing it with RAMP1, we highlight that not only is the RAMP essential for CLR function and CGRP peptide family signalling in primary cell systems but that RAMPs direct the pattern of agonist bias observed. Furthermore, we document previously unreported actions for the CGRP-based peptide agonists; AM2 emerges as an agonist uniquely biased to elevate calcium-mediated nitric oxide (NO) signalling while both CGRP and AM display distinct pro-proliferative effects in cardiovascular cells. The work we describe here reveals that GPCR agonist bias occurs naturally in human cells and plays fundamentally important physiological roles in providing unique functions to endogenous agonists.

Results

Endothelial cells exclusively express functional AM1 receptor. While there are many reports of biased agonism for GPCRs in recombinant systems (e.g.19–21), few examples have been documented in primary human cells. Given the reported roles of CGRP, AM and AM2 in the cardiovascular system we have focussed our studies upon these peptides, and their receptors in primary human umbilical vein endothelial cells (HUVECs), a well-established primary human vascular endothelial cell line. Initially, we determined that HUVECs express the AM1 receptor since we could only detect transcripts for CALCRL (gene for CLR) and RAMP2 (Fig. 1a). This was confirmed functionally, since when the endothelial cells were stimulated with agonists and cAMP accumulation quantified (after 30 min stimulation when the response had plateaued (Supplementary Fig. 1)), the rank order of potency was AM1 > AM2 > CGRP (Fig. 1b, and Supplementary Table 1). An important factor in confirming receptor-specific agonist bias is to ensure that competing receptors are not present in the system. The closely related calcitonin receptor not only interacts with RAMPs but also binds with high affinity. HUVECs do not appear to express the calcitonin receptor since we were unable to detect the presence of its transcript (Fig. 1a) or obtain a functional response upon application of two calcitonin receptor agonists (calcitonin or amylin) (Supplementary Fig. 1b). Furthermore, application of the selective AM1 receptor antagonist AM22–52 at 100 nM abolished agonist-induced cAMP accumulation while 100 nM olcegepant (a CGRP receptor-selective antagonist) had little effect (Supplementary Fig. 1c–h). Thus, based upon these data, we suggested that HUVECs specifically express the AM1 receptor alone and are a useful primary cell line with which to study potential endogenous agonist bias.

Endogenous agonist bias at the AM1 receptor. For studies of biased agonism, it is not simply the ability of different ligands to
activate the canonical second messenger pathway to varying extents that is important, but their ability to differentially activate a multitude of downstream pathways. Having established that HUVECs express only one of the receptor-RAMP complexes responsive to our three peptides: CGRP, AM and AM2 with the expected potency, we next sought to quantify the extent of endogenous agonist-induced biased signalling through the AM1 receptor at other pathways. Consistent with these previous reports in recombinant systems19 we were able to observe concentration-dependent increases in Ca\(^{2+}\) in HUVECs upon application of AM and AM2 but little or none with CGRP (Fig. 1c, and Supplementary Table 1). In contrast to the stimulation of cAMP, AM2 was more potent than AM suggesting that a non-cognate agonist can have a distinct and more potent effect than the cognate agonist at certain pathways endogenously. Importantly, all responses could be abolished with the co-treatment of the Gα\(_q/11/14\) inhibitor YM-25489024 (Supplementary Fig. 1i) thereby confirming CLR-based pleiotropy in primary endothelial cells.

We subsequently turned our attention to the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (assayed after 5 min stimulation, when the response had reached a plateau (Supplementary Fig. 1j)) where we found that, again, the ‘cognate’ agonist (AM) was not the most potent. Perhaps surprisingly, CGRP (the agonist reported to be the least potent at cAMP production at the AM1 receptor) was the most potent at stimulating ERK1/2.

Fig. 1 CGRP family peptide signalling bias in HUVECs. a Expression of CALCR, CALCRL, RAMP1, RAMP2, and RAMP3 genes in HUVECs. Data normalised to GAPDH expression. n = 3 independent experiments. b–f Dose–response curves were constructed for HUVECs stimulated with CGRP, AM or AM2 and the cAMP levels quantified relative to forskolin (100 μM) (n = 7) (b), mobilisation of Ca\(^{2+}\), relative to ionomycin (10 μM) (n = 6) (c), intracellular ERK1/2 phosphorylation relative to PMA (10 μM) (n = 4) (d), total nitric oxide production relative to acetylcholine (10 μM) (n = 3) (e), and extent of cell proliferation (after 72 h) relative to vector treated control and VEGF (n = 3–6) (f). Data are analysed using a three-parameter non-linear regression curve or the operational model of receptor agonism27. g–h Signalling bias plots were calculated as ΔLog(τ/K\(_A\)) (g) or ΔΔLog(τ/K\(_A\)) (h) for each agonist and for each signalling pathway. Determination of values requires normalisation to a reference agonist (AM) alone in (g), while for (h) values were normalised to both a reference agonist (AM) and a reference pathway (cAMP). i Heatmaps representing the signalling properties between HUVEC and HUAEC cells for potency, effector maximum and the transducer coefficient. All data represent mean ± SEM for n repeats. j Representation of the signalling outcomes as a result of AM-mediated receptor activation in a HUVEC. Solid arrows indicate known pathways. Dashed arrows represent possible pathways.
phosphorylation (Fig. 1d and Supplementary Table 1). Thus, despite this being designated an AM1 receptor, it is CGRP and not AM that produces physiologically relevant signalling via the ERK$_{1/2}$ pathway.

**Physiological consequences of CGRP-based peptide agonist bias in primary endothelial cells.** As we were exploring the AM1 receptor in its native environment, we sought to discover whether the distinct patterns of agonist bias we have observed with CGRP, AM and AM2 were reflected further downstream in physiologically relevant outputs. We considered two potential physiological outcomes with important therapeutic potential – the generation of NO (a vital modulator of vascular homeostasis) and cell proliferation. NO, generated through endothelial NO synthase in endothelial cells$^{25}$, promotes vasorelaxation/dilation$^{26}$. In HUVECs we observed that all three agonists could evoke NO synthesis in the order of potencies AM2 > AM > CGRP (Fig. 1e and Supplementary Table 1) although both AM and CGRP were partial agonists for this pathway with the potencies closely resembling the trends observed for Ca$^{2+}$ mobilisation. Indeed, a direct correlation between Ca$^{2+}$ mobilisation and NO production in endothelial cells was confirmed through the application of YM-254890 which abolished all NO release (Supplementary Fig. 1k). Such observations are consistent with the role of increases of Ca$^{2+}$ concentrations leading to endothelial NO synthase stimulation$^{25}$ but to the best of our knowledge, these have not been demonstrated previously for AM2.

Beyond NO production we also measured the long-term cell proliferation (72 h) response to the three peptides in HUVECs. Here CGRP most potently promoted cell growth (Fig. 1f and Supplementary Table 1). This is consistent with the data we describe for phosphorylation of ERK$_{1/2}$ suggesting proliferation is not mediated via a cAMP-dependent pathway. This was further corroborated by the observation that application of the non-selective adenyl cyclase activator forskolin induced a concentration-dependent inhibition of cell proliferation. Together, these data suggest that CLR exerts important cellular effects in a G$_{a_{i}}$-independent manner thus unveiling previously undocumented abilities for CGRP to promote proliferation in human cells through the AM1 receptor.

Whilst the differences in orders of potency seen with cAMP, Ca$^{2+}$, and ERK$_{1/2}$ provide strong evidence for bias, to formally confirm this and to remove potential confounding issue of system bias (which may arise due to the differential expression of signalling components or cofactors in the cellular background of choice) we fitted our HUVEC data with the operational model of receptor agonism$^{27}$ (Fig. 1g, h and Supplementary Table 1). This gives the transducer coefficient Log($\tau$/K$_{i}$); effectively the efficacy of an agonist to produce a given response normalised to its functional affinity. Agonist bias is calculated by computing $\Delta$Log ( $\tau$/K$_{i}$): the difference in transducer coefficient for each response compared to AM (Fig. 1h) and then the bias factor, $\Delta$Log($\tau$/K$_{i}$), where there is a second round of normalisation with respect to the cAMP pathway as well as AM (Fig. 1h). This analysis reinforced the notion that AM2 is biased towards Ca$^{2+}$ mobilisation and NO production while CGRP favours pERK$_{1/2}$ activation and cell proliferation.

We next wondered if the patterns of AM1 receptor bias applied to other endothelial cell lines. We performed the same panel of assays using human umbilical artery endothelial cells (HUAECs) which also solely express transcripts for RAMP2 and CALCRL (Supplementary Fig. 2a–h, Supplementary Table 1). Indeed we were able to demonstrate a strong similarity in the signalling profiles between the two endothelial cells across the five different pathways (Fig. 1i and Supplementary Fig. 3a, b) with significant correlations in potency (Supplementary Fig. 3c; r = 0.73–95% confidence interval 0.35–0.90; p = 0.0019) and the transducer coefficient (Fig. 1i, Supplementary Fig. 3c; $\tau$/K$_{i}$; r = 0.94–95% confidence interval 0.84 to 0.98; p < 0.0001 (to 4 decimal places)) suggesting primary endothelial cells share common AM1 receptor signalling properties (Fig. 1j).

**AM1 receptor-mediated cAMP accumulation and pERK$_{1/2}$ activation exemplify agonist bias.** The mechanism by which adenyl cyclase is regulated involves competition between G$_{a_{i}}$ (activation) and members of the G$_{i/o}$ subunits (inhibition) family of G proteins. Semi-quantitative RT-PCR in HUVECs and HUAECs revealed the presence of the same Ga subunits (Supplementary Fig. 3d–e) and $\beta$-arrestins in the two cell lines. We and others have documented how the AM1 receptor (analogous to other class B1 GPCRs) couples to the inhibitory G proteins$^{19,28,29}$ although this is often observed in overexpression systems and is cell type dependent. Application of pertussis toxin (PTX), which ADP-ribosylates the inhibitory G proteins (except for G$_{z}$), to the HUVECs revealed a dose-dependent increase in cAMP accumulation (Fig. 2a) and suppression of ERK$_{1/2}$ phosphorylation upon application of CGRP and AM2 but not AM (Fig. 2b). These data, consistent with our previously reported work$^{19}$ suggests that only the non-cognate agonists (CGRP and AM2) can recruit G$_{i/o}$ proteins to the CLR, and, particularly in the case of CGRP, the purpose of this is to bias the response away from cAMP and towards other pathways such as pERK$_{1/2}$.

This did, however, pose the question as to how AM modulates the pERK$_{1/2}$ response? Inhibition of protein kinase A had no effect (Fig. 2c) however antagonism of G$_{q/11/14}$ signalling reduced the potency of AM-mediated pERK$_{1/2}$ activation (Fig. 2d). More strikingly, inhibition of the exchange proteins directly activated by cAMP 1/2 significantly attenuated both the potency (p = 0.0078) and magnitude of the maximal response (Fig. 2e). Taken together, these data highlight the wide array of different G protein couplings and their interlinking actions upon downstream signalling events for the AM1 receptor. These couplings have not been engineered so are not enhanced by overexpression artefacts and thereby represent pure endogenous agonist bias.

**RAMP isoforn is essential for CLR-mediated agonist bias.** One of the advantages of using recombinant cell lines is the ability to switch the expressed GPCR or RAMP to observe effects on agonist bias. However, these recombinant systems do not allow for observations of physiological bias. Thus, we next sought to determine the effects of CGRP-based agonist bias in primary cells where the endogenous RAMP had been switched using gene deletion followed by lentiviral reintroduction in HUVEC cells. We used lentiviral CRISPR-Cas9 to knockout the RAMP2 gene from HUVECs using a pooled sgRNA strategy of using three sgRNAs in separate lentivirus (Supplementary Fig. 4a) which were selected using a puromycin resistance cassette (Supplementary Fig. 4b) to increase our efficiency of editing (95% as confirmed by Sanger sequencing (Supplementary Fig. 4c, d, Supplementary data 3, 4) and TIDE$^{30}$ analysis). We confirmed the loss of RAMP2 by qRT-PCR (Supplementary Fig. 4e) although the expression of the Ga subunits the $\beta$-arrestins remained consistent with wild type HUVECs (Supplementary Fig. 4f). The rate of proliferation of the cells was unchanged (Supplementary Fig. 4g) but as expected, there was no longer any functional response following stimulation with all three GCRP-based agonists (Supplementary Fig. 4h–l). We next introduced, using lentiviral overexpression and blasticidin selection, the open reading frame of RAMP1 into our HUVEC/RAMP2 cell line to, in effect, switch the expressed GPCR from the AM1 receptor to
CGRP, AM and AM2 confi of ERK1/2 phosphorylation in response to stimulation by CGRP, AM, and AM2 with and without PTX treatment relative to PMA (10 μM) (n = 4). b-e Characterisation of ERK1/2 phosphorylation in response to stimulation by CGRP, AM, and AM2 with and without PTX treatment relative to PMA (10 μM) (n = 4) (b), with/without Rp-8-Br-cAMPS (10 μM) (n = 3) (c), with/without YM-254890 (100 nM) relative to PMA (10 μM) (n = 3) (d) and with/without ESI-09 (100 μM) (n = 3) (e). Data are analysed using a three-parameter non-linear regression curve. All data represent mean ± SEM of n independent experiments. Statistical significance determined compared to control using an unpaired Student’s t test with Welch’s correction (*p < 0.05; **p < 0.01; ***p < 0.001). NS denotes no statistical significance observed. Rows show pEC50 and vertical arrows show Emax statistical significance.

Fig. 2 Non-cognate Go couplings at the AM1 receptor complex modulates cAMP accumulation and ERK1/2 phosphorylation. a Characterisation of cAMP accumulation in response to stimulation by CGRP, AM, and AM2 with and without PTX treatment relative to forskolin (100 μM) (n = 4). b-e Characterisation of ERK1/2 phosphorylation in response to stimulation by CGRP, AM, and AM2 with and without PTX treatment relative to PMA (10 μM) (n = 4) (b), with/withoutRp-8-Br-cAMPS (10 μM) (n = 3) (c), with/without YM-254890 (100 nM) relative to PMA (10 μM) (n = 3) (d) and with/without ESI-09 (100 μM) (n = 3) (e). Data are analysed using a three-parameter non-linear regression curve. All data represent mean ± SEM of n independent experiments. Statistical significance determined compared to control using an unpaired Student’s t test with Welch’s correction (*p < 0.05; **p < 0.01; ***p < 0.001). NS denotes no statistical significance observed. Rows show pEC50 and vertical arrows show Emax statistical significance.

the CGRP receptor. mRNA levels were quantified demonstrating successful introduction of a high level of RAMP1 expression (Fig. 3a). We next performed cAMP accumulation assays using CGRP, AM and AM2 confirming that a functional CGRP receptor was formed in these modified HUVECs (Fig. 3b and Supplementary Table 2). Reassuringly, we now observed that CGRP was the most potent agonist for the stimulation of cAMP—as expected for a cell line expressing the CGRP receptor (CLR-RAMP1). Perhaps more interestingly, CGRP was also the most potent at mobilising Ca2+ (Fig. 3c and Supplementary Table 2) and this was also the case in the associated NO production (Fig. 3d and Supplementary Table 2). Comparison of the ERK1/2 phosphorylation (Fig. 3e and Supplementary Table 2) highlighted that AM was now the most potent agonist; a clear switch from wild type HUVEC cells where CGRP was the most potent. This followed to proliferation where AM was also the most potent ligand, although both AM2 and CGRP could also promote growth (Fig. 3f and Supplementary Table 2) and contrasted with the wild type HUVECs where neither could cause proliferation. Thus switching the RAMP in the HUVEC cell line appeared to have a dramatic effect on the agonist bias observed and the functional consequence (Fig. 3g, h and Supplementary Table 2)—beyond just cAMP accumulation as would be expected. However, it should be noted that as RAMP1 expression was high we should be cautious in our direct comparisons between the wild type HUVECs and our RAMP1-HUVEC cell line.

Endogenous agonist bias at the CGRP receptor in primary human cardiac myocytes. To provide a comparison for RAMP1-HUVEC signalling with a primary cell line we turned to primary human cardiomyocytes (HCMs) since these cells only expressed CLR and RAMP1 (Fig. 4a); analogous to endothelial cells, HCMs do not express a functional calcitonin receptor (Fig. 4a and Supplementary Fig. 5a). To confirm that the mRNA expression translated to functional receptor expression we performed cAMP accumulation assays for the CGRP family of peptides (Fig. 4b and Supplementary Table 2). Here, CGRP was the most potent agonist followed by AM2 and AM, a pattern consistent with the expression of the CGRP receptor20 (also confirmed by application of 100 nM olcegepant to inhibit cAMP accumulation for all three agonists while 100 nM AM2–52 had little effect (Supplementary Fig. 5b–g)). Although Gai has previously been suggested to be important for PTX-sensitive effects from CLR19, upon application of PTX to HCMs we were unable to observe any change in the potency or maximal signalling for any of the three peptide agonists (Supplementary Fig. 5h–j), perhaps because the transcript for GNa12 (Gαi2) was lower than in the endothelial cells (Supplementary Fig. 5k). In contrast to the wild type HUVECs but analogous to the RAMP1-HUVECs cells, not only was CGRP able to stimulate Gα11/14-mediated-Ca2+ mobilisation in HCMs, but it was the most potent agonist (Fig. 4c and Supplementary Table 2). This did not directly translate to Gα11/14-mediated-NO production (Fig. 4d, Supplementary Fig. 5l, m and Supplementary Table 2) since all three agonists generated responses that were less distinct from each other. When quantifying ERK1/2 phosphorylation we observed that the cognate ligand (CGRP) was not the most potent (Fig. 4e and Supplementary Table 2) since all three agonists generated responses that were less distinct from each other. When quantifying ERK1/2 phosphorylation we observed that the cognate ligand (CGRP) was not the most potent (Fig. 4e and Supplementary Table 2). As in HUVECs, it was the least potent ligand at cAMP accumulation (AM) that was the most potent for ERK1/2 phosphorylation so demonstrating that AM can produce functionally relevant signalling responses at the CGRP receptor. The order of potency for the three agonists for ERK1/2 phosphorylation was replicated in
the long-term cell proliferation assays (Fig. 4f and Supplementary Table 2).

Analysis of the RAMP1-HUVEC signalling profile suggested a close overlap with the properties of HCMs for the five different signalling pathways as well as an opposing signalling profile to HUVECs (Fig. 5 and Supplementary Table 2). We confirmed this using correlation plots (Supplementary Fig. 6a–f) of both potency and transducer coefficient Log(τ/Kd), obtained from application of the operational model of receptor agonism and in bias factors (Fig. 5a, b, Supplementary Table 2, 3). Overall, our results show that the pattern of bias seen with the CGRP receptor is robust and is transferred between the HUVEC and HCM cell line backgrounds.

Discussion

We have shown that the CGRP family of endogenous peptides demonstrate biased agonism at the endogenous CLR in a physiological system; and that the RAMP expressed dictates the intracellular response and ultimately the physiological outcome. Many receptors have been shown to demonstrate agonist bias; but for the most part, this has been shown through synthetic ligands designed to target certain receptor pathways. We have now demonstrated the importance of studying GPCR second messenger signalling with the endogenous receptor in its native environment, with the distinct signalling patterns of AM2 we have uncovered providing a good example of this.

We have confirmed, using genome editing that, as anticipated by the co-expression models, that endogenous CLR is unable to function without RAMP expression. This provides, to the best of our knowledge, the first example of CRISPR-Cas9 interrogation of GPCR function in a primary cardiovascular cell. Furthermore, we have shown that the expression of a different RAMP in the HUVECs can switch the signalling bias of the CLR and associated peptide agonists, thus providing additional evidence that RAMP targeting could become a powerful therapeutic tool.

We have compared the pharmacology of these receptors in terms of cAMP accumulation to reports compiling multiple values from independent publications using the human receptor in transfected systems. It was reassuring therefore to observe that CGRP, AM, and AM2 displayed similar trends in cAMP potency at CGRP/AM1 receptors in the primary cells to those seen in recombinant co-expression studies, as well as in the gene-edited RAMP1-HUVECs which reflected the cAMP data from transfected systems (Fig. 5c). In addition, we have performed a comprehensive analysis of the mechanisms used by AM1 receptor to stimulate ERK1/2 phosphorylation in endothelial cells (Fig. 2). It is apparent that each agonist uses unique mechanisms to activate ERK1/2. For both CGRP and AM2 it is mediated by the co-expression models, that endogenous CLR is unable to function without RAMP expression. This provides, to the best of our knowledge, the first example of CRISPR-Cas9 interrogation of GPCR function in a primary cardiovascular cell. Furthermore, we have shown that the expression of a different RAMP in the HUVECs can switch the signalling bias of the CLR and associated peptide agonists, thus providing additional evidence that RAMP targeting could become a powerful therapeutic tool.

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Fig. 4 CGRP family peptide signalling bias in human cardiomyocytes. a Expression of CALCR, CALCRL, RAMP1, RAMP2, and RAMP3 genes in HCMs. Data normalised to GAPDH expression. n = 3 independent experiments. b-f Dose-response curves were constructed for HCMs stimulated with CGRP, AM or AM2 and the cAMP levels quantified relative to forskolin (100 μM) (n = 6) (b), mobilisation of Ca²⁺ relative to ionomycin (10 μM) (n = 4) (c), total NO production relative to isoproterenol (10 μM) (n = 3) (d), intracellular ERK1/2 phosphorylation relative to PMA (10 μM) (n = 4) (e) and extent of cell proliferation (after 72 h) relative to vector treated control and VEGF (n = 5) (f). Data are analysed using a three-parameter non-linear regression curve or the operational model of receptor agonism. g, h Signalling bias plots were calculated as ΔLog(τ/Kₐ) (g) or ΔΔLog(τ/Kₐ) (h) for each agonist and for each signalling pathway. Determination of values requires normalisation to a reference agonist (AM) alone in (g), while for (h) values were normalised to both a reference agonist (AM) and a reference pathway (cAMP). All data represent mean ± SEM for n repeats.

We can also consider this work in the wider context of the organs and systems these cells are found in as this sheds light on some of the pathways, and involvement of bias in some of the established roles of CGRP family peptides in the cardiovascular system. It has long been recognised that all three peptides show pleiotropic signalling, activating multiple G proteins and signalling pathways, but it has previously not been possible to fit this into any framework. We suggest our current observations on RAMP-directed bias may assist with this. AM has a multitude of important roles in vascular homeostasis, one of which is regulating endothelial barrier function. It is thought to cause barrier stabilisation and protect against infection mediated functional protein disappearance, all brought about initially through CAMP production. This is supported by our work demonstrating AM produces a potent CAMP response and is biased towards this pathway. It is also well documented that AM is a potent vasodilator known to mediate some of its vasodilatory effects through NO release from vascular endothelial cells, which we have pharmacologically profiled here.

In contrast, the precise role of AM2, which is also found in endothelial cells, has been unclear. We now provide evidence that AM2 is a potent stimulator of Ca²⁺ mobilisation and NO synthesis. It is possible therefore that this plays a vital role at least in umbilical endothelial cell physiology, and indeed wider vascular physiology. Thus, the finding of AM2’s greater potency than AM at eliciting NO release via Ca²⁺ mobilisation may have great therapeutic potential.

Interestingly, in vascular endothelial cells CGPR inhibits adenylyl cyclase through Gᵣₛ and predominantly signals through pERK₁/₂, and proliferation. The link between pERK₁/₂ and...
cellular metabolism/proliferation is well established\textsuperscript{40–42}, as well as in endothelial cells specifically\textsuperscript{43–45}. We have shown that where an agonist has biased signalling towards ERK\textsubscript{1,2} phosphorylation, this is carried through to long term cellular proliferation. Importantly, this shows overall that two non-cognate ligands, often not considered important for receptor function, do in fact have important signalling and physiological roles/capabilities. In addition, we have demonstrated that endogenous pERK\textsubscript{1,2} can come from a variety of sources depending on the stimulating ligand. Together this shows that CLR initiates a multitude of intracellular pathways beyond simply G\textsubscript{s} and cAMP/protein kinase A in physiologically relevant cells. Therefore, our data adds further evidence that AM for AM\textsubscript{1} receptor and CGRP for CGRP receptor should only be considered the cognate ligands in terms of G\textsubscript{s}-mediated cAMP signalling, and when looking at the physiology of RAMP2 in endothelial cells and the vasculature as a whole, CGRP and AM\textsubscript{2} should be considered alongside AM for their different and potentially complementary roles.

In the heart, there are multiple reports that CGRP has a cAMP-mediated positive inotropic and chronotropic effect\textsuperscript{5,46,47}, and our data showing its strong response in cAMP accumulation assays on human myocytes (combined with its overall bias towards this pathway) supports this. There are contrasting reports in the literature over AM’s effect on heart contractility\textsuperscript{7,48} with some suggestions that it is a positive inotrope acting in the same cAMP driven manner as \(\beta\)-adrenoceptor agonists\textsuperscript{49}, while others report it having negative inotropic effects\textsuperscript{50,51}. Here we show that AM promotes a cAMP response through CGRP receptor in human cardiomyocytes, but it has weak potency. This may provide some context/explanation for the contradictory literature reports. Furthermore, our report has clearly revealed that cAMP is not AM’s primary signalling pathway in HCMs and that it is biased towards pERK\textsubscript{1,2} and cell proliferation rather than cAMP and positive inotropy. Nevertheless, evidence suggests that AM has an important role in the human heart. This includes the observed elevation of AM in the failing heart\textsuperscript{52}. Here, we have
utilised proliferating human ventricular myocytes in vitro and shown that AM (but not CGRP or AM2) exhibits signalling bias specifically towards pERK1/2 and enhancing proliferation in these cells. This work highlights AM as a peptide hormone that may promote cardiac regeneration naturally in vivo and provides a cellular mechanism for this. This may also explain the elevation of AM in heart failure6 and the clinical trial data showing that AM administration reduces infarct size53. For AM2, its effect on contraction of the heart is blocked by both inhibitors of both protein kinase A and C, suggesting multiple signalling pathways are activated by this peptide in vivo54. This pleiotropy in signalling pathways is also present in vascular cells, which are themselves far from homogeneous. This may be relevant to the heterogeneity observed in the actions of CGRP and for example, the role of NO or other signalling pathways in vasodilatation. Indeed, CGRP has been suggested, in earlier studies, to mediate endothelium-dependent relaxation involving NO via an unknown endothelial receptor55. We suggest the AM1 receptor would be a viable candidate for this ‘unknown’ receptor. It is also worth noting that changes in RAMP expression have been observed due to pathological changes in blood pressure and in response to drug noting that changes in RAMP expression have been observed due to pathological changes in blood pressure and in response to drug treatment. These will all have complex effects as a result of bias and depending on the expression of CGRP, AM and AM256.

It should be noted that not all studies reveal pleiotropic signalling for the CGRP family of peptides57. We suggest that there is cell membrane (e.g. lipid composition) and cell line-specific factors (e.g. expression of G proteins) that influence the observed bias. It is also important to consider the temporal aspects of signal bias; in this study we have generally measured responses at what seems to be their peak up to 30-min stimulations. However, different patterns of bias may be observed if other time points are selected, particularly where signalling from internalised receptors is important.

In summary, we have gone beyond previous studies in recombinant systems to observe agonist bias. While we have focused upon CLR-RAMP complexes, our ability to switch the RAMP means we are, in effect, switching the expressed receptor and, therefore our work, has general applicability to all GPCRs. Our data highlights how endogenous agonist bias can have profound consequences for the cell and how important cell background is in regulating this process. This work may even go as far as to suggest that to fully understand bias at a GPCR, it must be considered in its native environment. While our work takes an important step closer to understanding how the CGRP family of peptides and receptors function on a cellular level in the human cardiovascular system, it also highlights the importance of endogenous agonist bias as a concept and emphasises its long-term consequences for drug design.

Materials and methods

Cell culture. HUVECs and HUAECs (were both sourced from PromoCell, Germany; C-12250 and C-12252 respectively) were cultured in Endothelial Cell Growth Media (ECGM) (PromoCell). Human Cardiac Myocytes (PromoCell, C-12811) were grown in Cardiac Myocyte Growth Media (CMGM) (PromoCell). All cell lines were cultured in media containing 10% heat inactivated Fetal Bovine Serum (FBS) (Sigma, USA). Cells were grown in 25 cm² flasks or 75 cm² flasks depending on cell density required. They were passaged approximately 4 days depending on confluency with a final volume of 10 ml produced from 1 ml of the previous cell culture and 9 ml of the growth medium in 75 cm² flasks, 1 ml using 4 ml in 25 cm² flasks, and used from passage of the primary HUVECs (PromoCell). HUVECs with the RAMP2 gene knocked out were generated by CRISPR/Cas9 homology-directed repair as described previously58. We chose the HUVEC cell line with which to perform the gene editing since we have been able to grow HUVECs beyond passage 6 to develop the RAMP2 null cells. We used a pooled sgRNA strategy using three sgRNAs in separate lentivirus (Supplementary Fig. 4b) which were injected using a polybrein resistance cassette (Supplementary Fig. 4b) to increase our efficiency of editing. The sgRNA sequences were designed (5’-CCG TCCGGTTGAGCGCCGG-3’, 5’-TCCGGTGGAGCGCCGGG-3’, and 5’-CCCGGTCTCCTAGCCCGA-5’. 3’ was the Cas9 target in the human RAMP2 gene (Sigma, USA). All guides were delivered in the LV01 vector (U6-sgRNA: eff-puro-2A-cas9-2A-(RFP) vector provided by (Sigma-Aldrich, US). Sequences were verified by Sanger sequencing. The control cell line was established by transduction of LV01 vector not containing sgRNA targeted to RAMP2 gene. HUVECs were seeded in 6 well plates at a cell density of 160,000 cells/well and maintained at 37 °C in 5% CO2 with Complete Endothelial Cell Growth Media containing 100 μg/ml streptomycin (Sigma-Aldrich, US). 24 h after seeding virus containing individual sgRNA/Cas9 constructs were pooled and transduced into cells at a high multiplicity of infection of 10, ensuring that each cell is infected by several lentivirus and increasing the likelihood of achieving knockout. Transduction was performed in media containing 8 μg/ml Polybrene (Sigma, USA). Cells were maintained at 37 °C for 24 h, then treated with Puromycin (1 μg/ml) (Thermo Fisher Scientific, UK) for 3 days to select for transduced cells. Cells then cultured without puromycin and expanded before cells were collected for genotyping by Sanger sequencing, qRT-PCR, and functional assays. All data shown were from cells expanded from these colonies. Cells were collected for genotyping by qRT-PCR and expanded for functional assays. All ‘HVEC-RAMP1’ data shown were from cells expanded from these colonies.

Immunofluorescence. HUVEC cells were seeded in Cell Carrier Ultra 96-well plate (PerkinElmer, Boston, MA, US) at a cell density of 160,000 cells/well and maintained at 37 °C in 5% CO2 with Complete Endothelial Cell Growth Media containing 100 μg/ml streptomycin. Cells were cultured for 24 h then treated with puromycin (1 μg/ml) (Thermo Fisher Scientific, UK) for 6 days to select for transduced cells. Cells were collected for genotyping by qRT-PCR and expanded for functional assays. All ‘HVEC-RAMP1’ data shown were from cells expanded from these colonies.

Sequencing of genomic loci. Genomic DNA was extracted from virally transduced HUVEC cells by: collecting approximately 10,000 cells, washing in PBS (sigma-Aldrich, US) and then lysing with DirectPCR Lysis Reagent (Viagen Biotech, US) containing protease K (Quagen, Germany) at 0.4 mg/ml. The lysate was incubated at 55 °C for 4 h; 88 °C, for 10 min; 12 °C for 12 h. PCR reaction was then set up in (20 μl) as follows: 2X Flash Phusion PCR Master Mix (Thermo Fisher, US) (20 μl), forward primer (5'-AATTCCGGGACAGCTGCTGTT-3') (Eurogentec, Belgium) (1 μl)(10μm), reverse primer (5'-GACGACCCTCCGAAAATAGGC-3') (Eurogentec, Belgium) (1 μl)(10μm), DNA (100 ng/ml), ddH2O (7 μl). The product was amplified by PCR using the following programme: 98 °C, 1 min; 35 x (98 °C, 10s; 55 °C, 10s; 72 °C, 15s), 72 °C, 1 min; 4 °C, hold. PCR clean-up was performed prior to sequencing using the Illustra GFX PCR DNA and Gel-band Purification Kit (Illustra, Germany) according to the manufacturer’s instructions. Editing of RAMP2 gene was confirmed by Sanger sequencing (Eurofins–Supplementary Fig. 4c, d; Supplementary data 3, 4) and TIDE analysis59.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). HUVECs were cultured as above in Complete Endothelial Cell Growth medium and plated in a 24-well plate at 100,000 cells/well. Media was then removed, and cells were washed in PBS (Sigma, UK). RNA was extracted and genomic DNA eliminated using an RNA extraction kit (Quagen, Germany) as per the manufacturer’s instructions. The cDNA was prepared according to measuring absorbance at 260 and 280 nm (Nanodrop ND-1000 Spectrophotometer, NanoDrop technologies LLC, Wilmington DE USA). RNA was used immediately for the preparation of cDNA using the Multiscribe reverse transcriptase. For the preparation of cDNA 100 ng of RNA was reverse transcribed at 37 °C for 1 h. The final reaction volume was 25 μl: 2 μl of the cDNA, 2 μl random primer (Life Technologies, US), 1 μl Superscript II (Life Technologies, US). Sequences were verified by Sanger sequencing. Reactions were performed on a thermal Cycler as following: 25 °C, 10 min; 48 °C, 30 min; 95 °C, 5 min. cDNA was stored at −20 °C.

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For each independent sample, qPCR was performed using TaqMan Gene Expression assays according to the manufacturer’s instructions (Life Technologies, MA, USA). Taq-Man® GPPDH, CALCR, CALCRL, RAMP1, RAMP2, RAMP3, all Ga subunits and β-arrestins were plated onto fast microamp plates containing 2 μl cDNA. 1 μl Taq-man probe, 10 μl Taq-man fast universal master mix (Applied Biosystems) and 10 μl dH2O, Oligonucleotides were used as described in Weston et al.\(^{29}\), \(^{30}\). For β-arrestin1 forward primer (5′-AACGACCCCGAGTGTTCAAG-3′) (Thermo Fischer, US) and reverse primer (5′-CTCATGGCTGCCATCACTG-3′), reverse primer (5′-ACAGAAGGCTGAACTCTAAAGG-3′). PCR reactions were performed on ABI 7900 HT real time PCR system (Thermo Fischer Scientific, US). The programme involved the following stages: 50 °C, 2 min; 95 °C, 10 min, the fluorescence detection over the course of 40 (95 °C, 15 s; 60 °C, 1 min). Data are expressed as relative expression of the gene of interest to the reference gene GAPDH where: Relative expression = 2^(-Cq of gene of interest − Cq of GAPDH)).

In vivo assays

Measurement of intracellular cAMP. All primary cell lines were cultured as above. On the day of the experiment media was replaced with PBS, before being dissociated with Trypsin-EDTA 0.05% (Gibco, UK) and then resuspended in PBS/BSA (0.1%) (Sigma, UK). Cells were immediately plated for use in cAMP assays as per the manufacturer’s instructions, reagents used were provided by the LANCE® cAMP detection assay kit (PerkinElmer, Boston, MA, USA), in 384 well optiplates (PerkinElmer Boston, MA, USA) at 2000 cells/well in 5 μl aliquots\(^{29}\), Human (h) eGCRP, hAM and hAM2 (Bachem, Switzerland) were diluted in PBS/BSA (0.1%) with 250 μM 3-Isobutyl-1-methylxantine (Sigma, UK), and used from 10 μM to 100 μM. Cells were incubated with compound for 30 min prior to adding detection buffer\(^{28}\),\(^{60}\). Plates were incubated for a further 60 min (room temperature) and then read on a plate reader (Mithras LB 940 microplate reader (Berthold technologies, Germany)). All responses were normalised to 100μM forskolin (Tocris, UK). Antagonist studies were performed in the same way through co-stimulation of the relevant concentration. Alongside control-treated cell. Experiments with PTX (Sigma, UK) required pretreatment (16 h) prior to assay. Cells were pre-treated with 100 nM YM-254890 (Alpha Laboratories, UK) (30 min), cells were then plated on 384 well plates, on the day of the experiment media was replaced with serum free media and used from 10 pM to 10 μM isoproterenol63,64. HCM responses were normalised to 10 μM acetylcholine. \(^{62}\) HCm responses were normalised to 10 μM isoproterenol63,64.

Statistics and reproducibility. All sample sizes were determined, and data analysed, in accordance with the guidelines described by Curtis M et al.\(^{65}\). All experiments were appropriately controlled using ‘system pathway’ agonists. If any of these pathway controls generated inappropriate responses, then the entire data set was removed from analysis. Tolerance for variation was <3-fold changes in potency for the system parameters tested. Data analysis for CAMP accumulation, Ca\(^{2+}\), mobilisation, NO accumulation, pERK1/2 activation, and cell proliferation analyses were performed in GraphPad Prism 8.4 (GraphPad Software, San Diego). Data were fitted to obtain concentration–response curves using either the three-parameter logistic equation using to obtain values of Emax and pEC50 or the operational model of agonism\(^{27}\). Statistical differences were analysed using one-way ANOVA followed by Dunnett’s post-hoc (for comparisons amongst more than two groups) or unpaired Student’s t test with Welch’s correction (for comparison between two groups). To account for the day-to-day variation experienced from the cultured cells, we used the maximal level of CAMP accumulation from cells in response to 100 μM forskolin stimulation was used as a reference, 10 μM Ionomycin for Ca\(^{2+}\), assays, 10 μM phosphor 12-myristate 13-acetate (PMA) for pERK1/2\(^{148}\). Emax values from these curves are reported as a percentage of these controls, and all statistical analysis has been performed on these data. Where appropriate the operational model for receptor agonism\(^{27}\) was used to obtain efficacy (τ) and equilibrium dissociation constant (Kd) values. In both cases, this normalisation removes the variation due to differences in days but retains the variance for control values. The means of individual experiments were combined to generate the curves shown. Having obtained values for τ and Kd these were then used to quantify signalling bias as the change in Log(Kd/τ)\(^{15,19}\). Error for this composite measure was propagated by applying Eq. 1.

\[
\text{Pooled SEM} = \sqrt{\left(\text{SEM}_M\right)^2 + \left(\text{SEM}_B\right)^2}
\]

Where, SEM\(_M\) and SEM\(_B\) are the standard error of measurement A and B. Correlations between pEC50 values and transducer coefficients Log(Kd/τ) were assessed by scatter plot and Pearson’s correlation coefficient (r) was calculated with 95% confidence interval.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Sequencing data is available in Supplementary Data 3, 4. Source data for the graphs and charts in the main figures are given as Supplementary Data 1, 2 and 5–7. Any remaining information including the primary data for all pharmacological investigations is available from the corresponding authors on reasonable request.

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Author contributions
D.R.P., A.M., M.W. and G.L. conceived and designed the research; A.J.C., T.V. and D.S. performed the experiments; A.J.C., N.M. and D.G. designed the CRISPR-Cas9 experiments, A.J.C., G.L., M.H. and M.W. analysed data; A.J.C., M.H. and G.L. wrote the paper, D.R.P., A.M., M.W. and D.G. revised and edited the paper.

Competing interests
The authors M.W., N.M. and D.G. declare the following competing interest: they are employees of, and shareholders in, AstraZeneca. The remaining authors declare no competing interests.

Additional information
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