Adrenomedullin and calcitonin gene-related peptide receptors in endocrine-related cancers: opportunities and challenges

Debbie L Hay, Christopher S Walker and David R Poyner

School of Biological Sciences and Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland 1142, New Zealand
1School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK
(Correspondence should be addressed to D R Poyner; Email: d.r.poyner@aston.ac.uk)

Abstract

Adrenomedullin (AM), adrenomedullin 2 (AM2/intermedin) and calcitonin gene-related peptide (CGRP) are members of the calcitonin family of peptides. They can act as growth or survival factors for a number of tumours, including those that are endocrine-related. One mechanism through which this occurs is stimulating angiogenesis and lymphangiogenesis. AM is expressed by numerous tumour types and for some cancers, plasma AM levels can be correlated with the severity of the disease. In cancer models, lowering AM content or blocking AM receptors can reduce tumour mass. AM receptors are complexes formed between a seven transmembrane protein, calcitonin receptor-like receptor and one of the two accessory proteins, receptor activity-modifying proteins (RAMPs) 2 or 3 to give the AM1 and AM2 receptors respectively. AM also has affinity at the CGRP receptor, which uses RAMP1. Unfortunately, due to a lack of selective pharmacological tools or antibodies to distinguish AM and CGRP receptors, the precise receptors and signal transduction pathways used by the peptides are often uncertain. Two other membrane proteins, RDC1 and L1/G10D (the ‘ADMR’), are not currently considered to be genuine CGRP or AM receptors. In order to properly evaluate whether AM or CGRP receptor inhibition has a role in cancer therapy, it is important to identify which receptors mediate the effects of these peptides. To effectively distinguish AM1 and AM2 receptors, selective receptor antagonists need to be developed. The development of specific CGRP receptor antagonists suggests that this is now feasible.

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Introduction

Adrenomedullin (AM) is a 52 amino acid peptide, which acts as a paracrine factor in many tissues in the body, most notably the cardiovascular system where it is produced by endothelial cells and causes vasodilation (Garcia et al. 2006). It belongs to the same peptide family as amylin, calcitonin and calcitonin gene-related peptide (CGRP), a 37 amino acid neuropeptide that is especially abundant in the sensory nervous system (Brain & Grant 2004). Another closely related peptide is adrenomedullin 2 (AM2; Takei et al. 2004), also known as intermedin (Roh et al. 2004). These peptides utilise the same G-protein-coupled receptor (GPCR), known as the calcitonin receptor-like receptor (CLR or CRLR). The pharmacology of this receptor is determined by three accessory proteins known as receptor activity-modifying proteins (RAMPs).

Although AM first attracted interest as a potent vasodilator, it soon became apparent that it had a number of other actions, such as to promote cell growth and survival and also to encourage both blood and lymph vessel formation. Together, these activities suggest that AM might be an important factor in tumour growth (Zudaire et al. 2003b, Garcia et al. 2006, Nakamura et al. 2006, Nikitenko et al. 2006). There is now considerable support for this hypothesis; however, there are areas of confusion, especially regarding the receptors that mediate the
effects of AM. This review will particularly focus on the challenges associated with deciphering how AM as well as CGRP and AM2 act in endocrine-related and other cancers.

**AM and CGRP receptors**

AM exerts its actions through two cell-surface receptors (Poyner et al. 2002). These are multimeric complexes of RAMPs with CLR, a family B GPCR. CLR in complex with RAMP2 forms an AM1 receptor whereas CLR complexed with RAMP3 forms an AM2 receptor (Fig. 1). These are both potently activated by AM; CGRP binds to the AM2 receptor with around 50-fold lower affinity than AM but has a much lower affinity at AM1 receptors. The RAMP1/CLR combination forms the CGRP receptor, which is potently activated by CGRP; AM can also signal via this receptor, binding with around 10-fold lower affinity than CGRP (Poyner et al. 2002, Bailey & Hay 2006). AM2 may have similar pharmacology to AM but this is poorly explored (Roh et al. 2004, Takei et al. 2004, Qi et al. 2008). These receptors predominantly signal via activation of adenylate cyclase and increase intracellular cAMP. Other receptors for AM and CGRP have been proposed in the past; these will be discussed later in this review.

**Physiological actions of AM on angiogenesis and lymphangiogenesis**

AM has profound actions on the growth and development of both the blood and lymphatic vascular systems. Mice deficient in AM show reduced vascularisation (Iimuro et al. 2004). Furthermore, AM administration to adult mice increased the number of blood and lymphatic vessels at the site of an injury (Jin et al. 2008). In vitro, AM can act upon endothelial cells derived from both blood and lymphatic vessels to promote neovascularisation, proliferation and migration (Miyashita et al. 2003b, Schwarz et al. 2006, Jin et al. 2008). It has been proposed that AM has a role in vascular maturation (Iwase et al. 2005). Thus, there is great interest in the AM system as a novel and important angiogenic pathway (Kahn 2008). Mice with disruption of the genes encoding either AM, CLR or RAMP2 die in utero with vascular defects, and are characterised by embryonic lethality, thin blood vessel walls and significant abnormalities in their vascular systems (Caron & Smithies 2001, Shindo et al. 2001, Ando & Fujita 2003, Dackor et al. 2006, 2007). The strikingly similar phenotype of all three of these mouse strains may be explained by the abnormalities in the blood and lymphatic vasculature (Fritz-Six et al. 2008, Ichikawa-Shindo et al. 2008). Interestingly, mice that lack RAMP3 do not show obvious changes in their vasculature and live to old age but have abnormalities in weight control (Dackor et al. 2007). This indicates that the two AM receptors have different functions.

**AM in cancer**

AM expression is widely distributed throughout the body, principally due to its production by endothelial cells. In several different tumour types, it has been reported that AM levels are strongly upregulated; especially in hypoxic environments (Table 1). These include endocrine or endocrine-related tumours where the tumours involve hormone-secreting cells

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**Figure 1** Composition of CGRP and AM receptors. CLR is the seven transmembrane (TM) protein. RAMP1 is shown in black, RAMP2 in dark grey and RAMP3 in white. ECD, extracellular domain; ICD, intracellular domain.
AM can act directly to stimulate cell growth and inhibit apoptosis in a variety of tumour cells (Forneris et al. 2001, Martinez et al. 2002, Albertin et al. 2005). AM can change the phenotype of cells, leading them to exhibit tumourigenic behaviour, for example, in prostate cancer, AM promotes the appearance of a neuroendocrine phenotype in cells (Martinez et al. 2002, Berenguer et al. 2008). AM may promote tumour blood and lymphatic angiogenesis, providing a supply of nutrients and oxygen to the tumour but also providing a means for tumour cells to spread (Garayoa et al. 2000, Fritz-Six et al. 2008, Ichikawa-Shindo et al. 2008). It is likely that this effect is mainly mediated by AM receptors on endothelial cells, although it has been reported that AM can activate mast cells in a receptor-independent manner to stimulate angiogenesis (Zudaire et al. 2006). AM may also reduce the effectiveness of the immune system to destroy tumours, by decreasing the expression of pro-inflammatory cytokines and inhibiting the activation of the alternative complement pathway by binding to complement factor H (Zudaire et al. 2003a).

The major sources of AM relevant to tumours are from the tumour cells themselves (Table 1) and the vascular endothelium. However, other cells that may be found associated with tumours such as mast cells can also produce the peptide (Zudaire et al. 2003b). Furthermore, AM signalling is enhanced under hypoxic conditions, chiefly via hypoxia-inducible factor-1α (HIF-1α). This is a transcription factor produced in response to hypoxia; it stimulates synthesis of both AM and CLR via hypoxic response elements located on their respective genes (Nikitenko et al. 2006). As solid tumours are typically hypoxic, they will be encouraged to produce even more AM and to respond to it with greater sensitivity.

### Table 1 The role of adrenomedullin (AM) in endocrine-related and other cancers

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>AM expression</th>
<th>Receptor expression</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Hepatic cancer</td>
<td>+</td>
<td>ND</td>
<td>Nakata et al. (2008)</td>
</tr>
<tr>
<td>Renal cancer</td>
<td>+</td>
<td>ND</td>
<td>Michelsen et al. (2006)</td>
</tr>
<tr>
<td>Thyroid</td>
<td>(CGRP)(^a)</td>
<td>ND</td>
<td>Zajac et al. (1986) and Pacini et al. (1991)</td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>ND</td>
<td>Martinez et al. (1995), Miller et al. (1996) and Buyukberber et al. (2007)</td>
</tr>
<tr>
<td>Breast</td>
<td>+</td>
<td>AM binding(^b), RAMP3</td>
<td>Martinez et al. (1995), Miller et al. (1996), Oehler et al. (2003) and Brekhman et al. (2010)</td>
</tr>
<tr>
<td>Gastro-entero pancreatic neuroendocrine</td>
<td>+</td>
<td>ND</td>
<td>Pavel et al. (2006)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>+</td>
<td>CLR, RAMPs 1, 2, 3</td>
<td>Ishikawa et al. (2003) and Ramachandran et al. (2007)</td>
</tr>
<tr>
<td>Pancreatic insulinoma</td>
<td>+</td>
<td>ND</td>
<td>Letizia et al. (2001)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>+</td>
<td>ND</td>
<td>Martinez et al. (1995) and Liu et al. (2009)</td>
</tr>
<tr>
<td>Endometrial</td>
<td>+</td>
<td>CLR, RAMP1 (myometrium, vascular smooth muscle) CLR, RAMP2 (uterine smooth muscle)</td>
<td>Michishita et al. (1999) and Nikitenko et al. (2001)</td>
</tr>
<tr>
<td>Prostate</td>
<td>+</td>
<td>CLR, RAMPs 2, 3</td>
<td>Rocchi et al. (2001), Calvo et al. (2002) and Berenguer et al. (2008)</td>
</tr>
<tr>
<td>Pituitary adenoma</td>
<td>+</td>
<td>ND</td>
<td>Knerr et al. (2001)</td>
</tr>
<tr>
<td>Somatotropinoma</td>
<td>+</td>
<td>ND</td>
<td>Knerr et al. (2001)</td>
</tr>
<tr>
<td>Prolactinoma</td>
<td>+</td>
<td>ND</td>
<td>Knerr et al. (2001)</td>
</tr>
<tr>
<td>Adrenocortical carcinoma</td>
<td>+</td>
<td>CLR, RAMP3</td>
<td>Albertin et al. (2005)</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>+</td>
<td>CLR, RAMPs 1, 2, 3</td>
<td>Zudaire et al. (2003a,b) and Thouennon et al. (2010)</td>
</tr>
<tr>
<td>Aldosteronoma</td>
<td>+</td>
<td>CLR, RAMPs 1, 2, 3</td>
<td>Albertin et al. (2001) and Forneris et al. (2001)</td>
</tr>
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\(^a\)CGRP expression.

\(^b\)\(^{125}\)I AM radioligand binding in MCF-7 cells.

This table is based on data from Zudaire et al. (2003b) but has been updated and modified with additional data. ND, not done.

(e.g. pancreatic insulinoma) or develop a neuroendocrine phenotype (prostate cancer; see below). AM does not cause cancer but can contribute to its pathogenesis in several ways. The overall scheme of activity of AM in the tumour microenvironment is shown in Fig. 2. AM can act directly to stimulate cell growth and inhibit apoptosis in a variety of tumour cells (Forneris et al. 2001, Martinez et al. 2002, Albertin et al. 2005). AM can change the phenotype of cells, leading them to exhibit tumourigenic behaviour, for example, in prostate cancer, AM promotes the appearance of a neuroendocrine phenotype in cells (Martinez et al. 2002, Berenguer et al. 2008). AM may promote tumour blood and lymphatic angiogenesis, providing a supply of nutrients and oxygen to the tumour but also providing a means for tumour cells to spread (Garayoa et al. 2000, Fritz-Six et al. 2008, Ichikawa-Shindo et al. 2008). It is likely that this effect is mainly mediated by AM receptors on endothelial cells, although it has been reported that AM can activate mast cells in a receptor-independent manner to stimulate angiogenesis (Zudaire et al. 2006). AM may also reduce the effectiveness of the immune system to destroy tumours, by decreasing the expression of pro-inflammatory cytokines and inhibiting the activation of the alternative complement pathway by binding to complement factor H (Zudaire et al. 2003a).
AM over-expression by tumours and in many cases the data are unavailable. This is because the majority of tissues normally express AM and appropriate comparisons between normal tissue or cells and tumours can seldom be made.

**Disease models**

Xenografts or the injection of tumour cells, which have been engineered to over-express AM into susceptible mice, can lead to tumour formation (Martinez *et al.* 2002, Oehler *et al.* 2002). Furthermore, agents that reduce the activity of AM such as the peptide antagonist AM22–52 and agents that bind directly to AM reduce endothelial cell proliferation, migration, angiogenesis, tumour vascularisation and tumour growth (Ishikawa *et al.* 2003, Miyashita *et al.* 2003a, Iimuro *et al.* 2004, Schwarz *et al.* 2006, Roldos *et al.* 2008, Tsuchiya *et al.* 2010). For example, tumour weight was reduced compared with control following intratumoural injection of AM22–52 in a mouse model of in vivo pancreatic cancer cell growth (Ishikawa *et al.* 2003). This suggests that the antagonist was able to reduce the activity of endogenous AM produced by the tumour or local blood vessels (Ishikawa *et al.* 2003). Moreover, antibodies to the AM1 and AM2 receptors inhibited migration and capillary tube formation of endothelial cells and the growth of human tumour xenografts but it is important to note that the specificity of these antibodies has not been properly reported (Fernandez-Sauze *et al.* 2004, Kaafarani *et al.* 2009).

**The roles of AM1 and AM2 receptors in tumour progression**

As noted above, the AM1 and AM2 receptors have distinct roles. Where information is available, it is the AM1 receptor that is most often implicated in the direct effects on growth and survival of tumour cells. RAMP2 is expressed by clear cell renal carcinoma cells, hepatocellular carcinoma cells and pancreatic tumour cells (Keleg *et al.* 2007, Park *et al.* 2008, Deville *et al.* 2009); it is also expressed in a number of endocrine-related and other cancers (Table 1). In some cases there is also expression of RAMP3, making it difficult to know which receptor is mediating the effects of AM; however, neither renal nor pancreatic cancer cells express RAMP3. CLR knockout mice, lacking the GPCR component of AM receptors, die in utero and display severe vascular defects (Dackor *et al.* 2006), whereas mice haplo-insufficient for CLR are relatively normal (Dackor *et al.* 2007). Only the RAMP2 knockout mouse shows a vascular phenotype.

**Clinical evidence**

There are now many studies that show an association between AM expression and cancer. These are predominantly studies where AM mRNA and/or protein expression have been determined in different tumour types and compared with normal tissue. In some studies, plasma AM concentrations have also been measured. These studies suggest that AM is over-expressed in numerous endocrine-related and other tumours including renal cell carcinoma, granuloma cell tumours, hepatocellular carcinoma, non-small cell lung carcinoma, bulky invasive squamous cell carcinoma and neuroendocrine tumours as shown in Table 1 (Li *et al.* 2003, Michelsen *et al.* 2006, Pavel *et al.* 2006, Buyukberber *et al.* 2007, Nakata *et al.* 2008, Liu *et al.* 2009). It is also possible that this apparent over-expression could occur through decreased disposal or enhanced processing of mature AM. Furthermore, in some tumours it is possible to correlate AM expression with disease progression. In breast cancer, AM plasma concentrations correlate with the presence of lymph node metastasis and in hepatocellular carcinoma, AM expression was positively correlated with invasion (Oehler *et al.* 2003, Park *et al.* 2008). In neuroendocrine cancers, AM expression correlates with tumour progression (Pavel *et al.* 2006). Interestingly, in primary aldosteronism due to adenoma, elevated AM levels return to normal after corrective surgery (Letizia *et al.* 1998). Thus, there is a significant body of evidence showing association of AM and its receptors with cancer. However, it should be noted that it is frequently very difficult to ascertain the true level of
(Fritz-Six et al. 2008, Ichikawa-Shindo et al. 2008). RAMP3 cannot compensate for RAMP2’s absence (Dackor et al. 2007). Aortic rings from mice with reduced RAMP2 expression show reduced angiogenesis (Ichikawa-Shindo et al. 2008). In endothelial cells over-expressing either RAMP2 or RAMP3, only the RAMP2 over-expressing cells show increased capillary formation (Ichikawa-Shindo et al. 2008). In a separate study, RAMP2 gene silencing in human saphenous vein endothelial cells reduced AM-stimulated proliferation and tube formation (Guidolin et al. 2008). Although the AM₁ receptor seems to be the likely candidate mediating the vascular effects of AM, the AM₂ receptor also appears to have actions in tumour biology. As noted above, in some tumours RAMP3 is expressed alongside RAMP2. RAMP3 mRNA expression is elevated in prostate carcinoma cells as opposed to those from prostate hypertrophy (Mazzocchi et al. 2004). In a study of renal tumours, RAMP3 elevation was reported in inflammatory cells associated with the tumour, whereas RAMP2 was found in the tumour cells themselves (Deville et al. 2009). This is consistent with a complex role of AM and its receptors, acting directly on the tumour itself and on its environment. In SW-13 cells, derived from an adrenocortical carcinoma, only CLR and RAMP3 are expressed, compared to all three RAMPs in normal adrenocortical cells; in this case, the growth-promoting effects of AM must be mediated via the AM₂ receptor (Albertin et al. 2005). It remains unresolved as to whether the loss of RAMP1 and 2 expressions in SW-13 cells is typical of adrenocortical tumour cells as a whole. Recently, inhibition of RAMP3 expression in breast cancer cells was shown to reduce tumour development (Brekhman et al. 2010).

Typically, both AM₁ and AM₂ receptors signal via stimulation of Gₛ and production of cAMP. It is perhaps not surprising that a number of studies have shown that AM can increase the activity of members of the MAP kinase family and protein kinase B/Akt. In some cases this is probably due to an initial increase in cAMP (Ouafik et al. 2009), but this cannot be assumed to apply to all situations.

CGRP in cancer

Although CGRP itself is not believed to cause cancer, it may also contribute to disease progression. In vivo, CGRP infusion increased implanted tumour volume (Toda et al. 2008). Furthermore, in a rat hindlimb model, ischaemic conditions increased CGRP content and CGRP over-expression enhanced angiogenesis (Zheng et al. 2010). In vitro, CGRP can increase cellular proliferation (Kawase et al. 2005, Kawanami et al. 2009), endothelial cell tube formation and migration (Zheng et al. 2010) and the invasiveness of prostate-cancer-derived cells by enhancing both cell mobility and haptotactic migration to fibronectin (Nagakawa et al. 1998, 2001). Further evidence for a role of CGRP in cancer comes from the study of CGRP knockout mice; implanted tumours displayed reduced tumour size and vascularisation compared to control mice (Toda et al. 2008). Furthermore, infusion of the CGRP receptor antagonist, CGRP₈–₃₇, mirrored the CGRP knockout model, with an implanted tumour displaying reduced growth and angiogenesis (Toda et al. 2008).

Elevated CGRP expression has been identified in both plasma and tumours from specific cancers; including small cell lung carcinomas (Kelley et al. 1994), prostate cancer (Suzuki et al. 2006), breast cancer (Papantoniou et al. 2007, 2010) and thyroid cancer (Pacini et al. 1991, 1992). In prostate cancer, serum CGRP correlated with high-grade/stage disease (Suzuki et al. 2006), however, hormone replacement therapy may interfere with CGRP’s use as a diagnostic marker (Suzuki et al. 2009). Interestingly, CGRP has been associated with several symptoms related to specific cancers. For example, in medullary thyroid cancer patients, elevated plasma CGRP is associated with increased heart rate (Rubinstein et al. 1992). Tumour-associated pain is one of the most debilitating symptoms of cancer and is typically associated with metastatic invasion of bone tissue. In a model of bone cancer invasion, hyperalgesia is associated with increased spinal cord CGRP content and subsequent irradiation treatment results in reduced hyperalgesia and spinal cord CGRP content (Park et al. 2005). In a mouse model, tumours associated with mechanical hyperalgesia were more densely innervated with CGRP expressing nerve fibres and less vascularised than tumours from non-hyperalgesic mice (Wacnik et al. 2005). Furthermore, intratumoural injection of CGRP₈–₃₇ partially blocked tumour-associated pain (Wacnik et al. 2005), suggesting that treatment with one of a new class of small molecule CGRP receptor antagonist (Doods et al. 2007), developed for migraine treatment, may reduce pain and improve the quality of life for some cancer sufferers. Interestingly, in one study, tumour implantation was shown to increase dorsal root ganglion (DRG) CGRP expression; following denervation, DRG CGRP expression was normalised in association with reduced tumour growth and angiogenesis (Toda et al. 2008).
The role of CGRP receptors in cancer

Although information is limited, several lines of evidence indicate that CGRP receptors may play distinct roles in cancer. The expression of CGRP receptor components and CGRP activity has been examined in several tumours and tumour-derived cell lines. For example, RAMP1 mRNA expression has been detected in benign and malignant pheochromocytomas (Thouennon et al. 2010), Conn’s adenoma (Forneris et al. 2001) and pancreatic cancers (Ramachandran et al. 2007).

CGRP receptors typically signal via stimulation of Gs and production of cAMP, however, several other pathways can be activated (Walker et al. 2010). In several studies, CGRP has been shown to increase proliferation by activating members of the MAP kinase family (Kawase et al. 2005, Kawanami et al. 2009) and increase vasodilation via cGMP and NO production (Brain & Grant 2004). The functional significance of the CGRP receptor in cancer is less clear than AM receptors.

AM2 (intermedin) and cancer

Since the identification of AM2 there has been considerable interest in elucidating its biological functions. As AM2 is capable of interacting with CGRP, AM1 and AM2 receptors (Roh et al. 2004), it is perhaps unsurprising that several actions attributed to CGRP and AM have been replicated by AM2. Although it is unlikely that AM2 causes cancer directly, AM2 may contribute to cancer progression through several mechanisms. AM2 has been shown to promote differentiation of cells; proangiogenic effects were observed in human umbilical vein endothelial cells (HUVECs), where AM2 increased vascular endothelial growth factor (VEGF) expression and VEGFR-2 activity (Albertin et al. 2010). Furthermore, AM2 stimulated cell migration and tube formation via extracellular signal related kinase (ERK) activation in cultured endothelial cells and increased capillary/arteriole density in an ischaemic hindlimb model (Smith et al. 2009). AM2 also displays anti-apoptotic properties (Pearson et al. 2009, Song et al. 2009). Interestingly, AM2 is also upregulated under hypoxic conditions in mouse lung, hepatocytes and endothelial cells at least in part via HIF-1α (Copple et al. 2009, Pfeil et al. 2009).

Proadrenomedullin N-terminal 20 peptide and cancer

Proadrenomedullin N-terminal 20 peptide (PAMP) is produced along with AM by processing of a precursor polypeptide. It is angiogenic at concentrations in the femtomolar range and its antagonist, PAMP₁₂₋₂₀, can reduce tumour growth in xenograph models (Martinez et al. 2004). The PAMP sequence is distinct from that of AM and it appears to act through distinct receptors (Kamohara et al. 2005). As such, a detailed consideration of it is beyond the scope of this review. However, its probable co-release with AM suggests that a combination of AM and PAMP antagonists may be useful in anti-angiogenesis therapy.

Identification of AM and CGRP receptors involved in tumour progression

From what has been said above, it is clear that AM and related peptides may promote tumour growth by a number of mechanisms and receptors. It is important to identify the receptors that are involved at each step, both to understand the biological processes involved and also to inform drug discovery programmes, given the potential of the AM and CGRP receptors as therapeutic targets. Unfortunately, this is far from simple. The main issues are considered below.

What constitutes an AM or a CGRP receptor?

Although it is clear that the AM and CGRP receptors are complexes between CLR and RAMPs, the early literature on this topic was misleading, due to the mistaken identification of two orphan GPCRs, RDC1 and L1/G10D as receptors for CGRP and AM respectively (Kapas & Clark 1995, Kapas et al. 1995). Regrettably, it has proved impossible to repeat the initial observations with either of these receptors (Kennedy et al. 1998, McLatchie et al. 1998). Consequently, the International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature subcommittee for receptors of the calcitonin family has determined that neither of these receptors should be considered as CGRP or AM receptors respectively (Poyner et al. 2002). We will consider some pertinent aspects of this matter further in this section.

L1/G10D, now known as GPR182, remains an orphan GPCR with no known ligand (http://www.iuphar-db.org/DATABASE/ObjectDisplayForward?id=146). Unfortunately, the original misidentification of this protein as an AM receptor continues to confuse a number of workers who refer to an ‘ADMR’ (AM receptor) based on L1/G10D (Ramachandran et al. 2007, 2009). It is particularly regrettable that the official gene name for L1/G10D has until recently been ADMR, perpetuating the unsubstantiated concept that this is a receptor for AM. The official gene name is now GPR182.
RDC1 was initially reported as a CGRP receptor; it has been reported that this observation could not be repeated (McLatchie et al. 1998). In the original publication, it was reported that RDC1 could also bind AM with low affinity (200 nM). This seems to have generated the notion that RDC1 is a putative AM receptor (Thouennon et al. 2010). There has been no independent verification that RDC1 can bind AM and until it can be demonstrated that RDC1 can produce a functional response inside a cell on binding AM, it cannot be considered as an AM receptor. There are reports in the literature that AM signalling and the presence of RDC1 are associated but this correlation does not mean that RDC1 is a receptor for AM (Autelitano & Tang 1999, Chakravarty et al. 2000, Ladoux & Frelin 2000, Sierro et al. 2007, Thouennon et al. 2010). There are many possible explanations, such as regulation of CLR and/or RAMP expression, for example. RDC1 has been considered a receptor for the chemokine SDF1 and has been renamed as CXCR7 (Balabanian et al. 2005), although the IUPHAR sub-committee on chemokine receptor nomenclature do not currently recommend this (http://www.iuphar-db.org/latestPairings.jsp). RDC1 was also once considered as a vasoactive intestinal peptide receptor and therefore there have clearly been problems with identifying its true function.

There remains the problem that some of the earlier literature on AM and cancer predates the resolution of the AM receptor problem (Miller et al. 1996, Martinez et al. 1997); there is a need to reconsider the nature of the AM receptors that were identified in some of these reports. It is recommended that IUPHAR guidelines should be followed when considering receptors for CGRP or AM; if possible, the molecular composition should be specified (i.e. CLR/RAMP1) to avoid ambiguity. In particular, the phrase ‘adrenomedullin receptors’ should not be used in overarching statements such as ‘adrenomedullin receptors were upregulated in cancer’ without defining the precise molecular components involved.

**Discriminating between the AM1, AM2 and CGRP receptors**

AM at pharmacological concentrations (> 10 nM) can activate the AM1, AM2 and CGRP receptors. It is possible to distinguish between CGRP and AM receptors pharmacologically. Traditionally, this has been done using peptide antagonists; CGRP<sub>8–37</sub> is selective for the CGRP receptors, AM<sub>22–52</sub> is selective for the AM1 and AM2 receptors (Poyner et al. 2002, Hay et al. 2003). If this approach is used, then ideally both antagonists should be compared to determine their relative potency. There are now non-peptide CGRP antagonists available such as BIBN4096BS and MK0974, these show much greater selectivity for the CGRP over AM receptors and provide a simpler approach to distinguish between the CGRP and AM receptors (Hay et al. 2002, Salvatore et al. 2008). The pharmacological discrimination between the AM<sub>1</sub> and AM<sub>2</sub> receptors is much harder. Although AM<sub>22–52</sub> shows a small degree of selectivity, the size of this makes it difficult to exploit (Hay et al. 2003).

If the pharmacological tools to distinguish between the AM<sub>1</sub> and AM<sub>2</sub> receptors are not currently available, how else may they be identified? The most reliable technique is to identify the mRNA, by any of the standard techniques that are available to do this. However, it is important to identify the receptor components in the specific cell types of interest to ensure that there is likely to be a direct relevance with tumour progression. Of course, there is not always a simple relationship between mRNA and protein expression. Indeed, it has sometimes been argued that low expression of CLR or RAMPs is a sign that these are not involved in an AM-mediated response (Thouennon et al. 2010). However, even if low mRNA can be equated with low expression of protein, it does not need high expression of a GPCR to produce a response. These receptors are capable of considerable signal amplification; thus, very low expression does not mean that the receptor could not function. There can be considerable variation in the amount of CLR expression that nevertheless produces CGRP or AM binding and function (Choksi et al. 2002).

An alternative to identifying the mRNA transcript is to use an antibody against the protein components, either CLR or the RAMPs. These are commercially available. However, the experience of the authors is that it is necessary to carry out rigorous controls to confirm their specificity. In particular, it is recommended that any antibody be compared on a western blot in control cells and cells that have been transfected with the desired component, to ensure that a product of the correct molecular weight is only present in the transfected cells. Furthermore, antibodies should ideally be tested in knockout tissues to confirm their specificity. Unfortunately, many published studies using commercially available antibodies do not appear to have performed these essential controls. When considering using any antibody that has been generated using a peptide, it is important to consider where this epitope is found in the intact protein complex (Zhao et al. 2006, Eftekhari et al. 2010).
The fact that RAMPs can also associate with other receptors must not be neglected (Christopoulos et al. 2003). This adds an additional complication to the situation.

As an alternative to the use of chemical antagonists, knockdown with siRNA may be used, if the system lends itself to this approach (Bouschet et al. 2005, Thouennon et al. 2010). This is most convincing when combined with one of the techniques described above to identify the component in the cells.

Signal transduction pathways used by AM-related peptides in tumour biology

There is a need to further delineate the signal transduction pathways used by AM and its relatives, in particular, to establish how far cAMP-independent mechanisms may be used. This may ultimately become important if pathway-selective antagonists for AM and CGRP are developed (Kenakin 2008). This work will need careful use of inhibitors of the different second messenger pathways.

Future perspectives

There seems little doubt the AM and related peptides are important factors in a number of endocrine-related tumours. There is now considerable evidence to implicate both direct actions on the tumour cells themselves and also indirect actions, particularly related to angiogenesis. More work is needed to establish the receptor mechanisms that are used by these peptides. Most work has been done for AM. The majority of therapeutic agents work at cell-surface receptors and so it is likely that this will be the most fruitful approach to blocking AM action, although it is possible to target AM itself through small molecule inhibitors or appropriate antibodies (Roldos et al. 2008). To target the individual receptors, there is an urgent need for high-affinity, selective AM receptor antagonists suitable for in vivo studies. Ideally, these should include the AM1 and AM2 receptor antagonists; there is also a case for combining these with another molecule that blocks the CGRP receptor activity to treat cancer. Antagonists that block the AM1, AM2 and CGRP receptors simultaneously may also prove useful as cancer therapies. Peptide antagonists of this nature have been developed (Robinson et al. 2009). Such antagonists are also likely to block any action of AM2. Initially, these agents need to be used in a wide range of in vivo models to examine the effectiveness of AM inhibition in treating tumours. It might be expected that they will be most effective in solid tumours where growth or survival of the cancer cells themselves is directly stimulated by AM or its relatives. In these cases, AM is likely to be promoting tumour progression both directly and indirectly (via effects on angiogenesis); thus, the antagonists will be able to block multiple processes. It is also important to understand exactly how AM might interact with other angiogenic molecules, to determine whether it can usefully be applied as part of a combination therapy. Given the number of factors that are involved in blood and lymph vessel growth this may be the most realistic option for AM antagonists. In parallel with these studies, the safety of AM antagonists needs to be evaluated in early phase clinical trials. The knockout studies have given us some pointers as to the physiological role of AM in mice; however, it is not always obvious how this might apply to humans. Good antagonists would provide the necessary experimental evidence.

Thus, the key to confirming the role of AM in cancer is likely to be the availability of appropriate antagonists. There are grounds for thinking that the discovery of these compounds might be feasible. A number of high-affinity CGRP antagonists are now available; these exploit a binding groove between CLR and RAMP1. A crystal structure of a bound antagonist at the CLR/RAMP1 complex has been solved (ter Haar et al. 2010). Although no structures of CLR/RAMP2 or RAMP3 are available, it is likely that these show similar folds to the CLR/RAMP1 complex, in particular, there are likely to be homologous pockets that can be exploited by antagonists.

Special comment is needed as to the role of CGRP in cancer. It is particularly intriguing that symptoms associated with certain tumours, especially pain, seem to correlate with elevated CGRP levels. Selective CGRP antagonists are available and are in clinical trials for migraine (Durham & Vause 2010); it would be particularly interesting to see whether these could also be used in cancer therapy.

Summary

There is significant evidence for a feed-forward mechanism involving AM in cancer. AM can be produced by tumours, especially under hypoxic conditions and stimulates blood and lymph vessel angiogenesis, promoting tumour growth and spread. To a greater or lesser extent, its actions may be shared between CGRP and AM2. The receptors that mediate the actions of CGRP and AM are complexes of CLR and RAMPs. The AM1 receptor (CLR/RAMP2) is particularly important in angiogenesis; the receptor
type(s) involved in the other tumourigenic actions of the peptides is less clear. There are significant technical challenges associated with identifying these receptors, although these are surmountable with careful experimentation and the inclusion of the appropriate controls. If new antagonists can be developed in the near future, then rapid progress could be made to establish the status of the individual peptides and receptors as drug targets in cancer treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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