

Themed Section: Secretin Family (Class B) G Protein-Coupled Receptors –  
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## REVIEW

# The pharmacology of Adrenomedullin 2/ Intermedin

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Adrenomedullin 2 (AM2) or intermedin is a member of the calcitonin gene-related peptide (CGRP)/calcitonin family of peptides and was discovered in 2004. Unlike other members of this family, no unique receptor has yet been identified for it. It is extensively distributed throughout the body. It causes hypotension when given peripherally, but when given into the CNS, it increases blood pressure and causes sympathetic activation. It also increases prolactin release, is anti-diuretic and natriuretic and reduces food intake. Whilst its effects resemble those of AM, it is frequently more potent. Some characterization of AM2 has been done on molecularly defined receptors; the existing data suggest that it preferentially activates the AM<sub>2</sub> receptor formed from calcitonin receptor-like receptor and receptor activity modifying protein 3. On this complex, its potency is generally equivalent to that of AM. There is no known receptor-activity where it is more potent than AM. In tissues and in animals it is frequently antagonised by CGRP and AM antagonists; however, situations exist in which an AM2 response is maintained even in the presence of supramaximal concentrations of these antagonists. Thus, there is a partial mismatch between the pharmacology seen in tissues and that on cloned receptors. The only AM2 antagonists are peptide fragments, and these have limited selectivity. It remains unclear as to whether novel AM2 receptors exist or whether the mismatch in pharmacology can be explained by factors such as metabolism.

### LINKED ARTICLES

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### Abbreviations

AC187, acetyl-(Asn<sup>30</sup>,Tyr<sup>32</sup>)-calcitonin<sub>8-32</sub>; AM, adrenomedullin; BIBN4096BS 1-piperidinecarboxamide, *N*-[2-[[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazoliny)-, [R-(R\*,S\*)]; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CT, calcitonin; ICC, immunocytochemistry; IMD, intermedin; RAMP, receptor-activity modifying protein; RT-PCR, reverse transcriptase-PCR

## Introduction

The peptide known either as adrenomedullin 2 (AM2) or intermedin (IMD) was independently discovered by two groups in 2004 (Roh *et al.*, 2004; Takei *et al.*, 2004a). It is most closely related to AM. In fish it is one of a family of five AM-related peptides, but in mammals, there is currently only evidence for widespread expression of AM and AM2; in some

mammals (such as ungulates and lower primates but not rodents or humans), the equivalent of fish AM5 is also found (Takei *et al.*, 2008).

AM2/IMD sits within the wider calcitonin (CT) gene-related peptide (CGRP)/CT family of peptides, which also includes amylin. It is expressed in both peripheral tissues and in the CNS; its effects generally resemble those of AM, but it is sometimes more potent and appears to have some unique actions (Taylor and Samson, 2005; Hashimoto *et al.*,

2007). This review considers the pharmacology of AM<sub>2</sub>/IMD, both on molecularly defined receptors and in native cells and tissues. It considers its specificity for known receptors belonging to the CGRP/CT peptide family and also evaluates the evidence that it may act on other receptors.

## Distribution of AM<sub>2</sub>/IMD

The distribution of AM<sub>2</sub>/IMD in cells and tissues overlaps with AM. Compared with AM, AM<sub>2</sub>/IMD is less widely distributed in mammals (Taylor *et al.*, 2005). Table 1 summarizes the distribution of AM<sub>2</sub>/IMD in organs and tissues of different species. Generally, this peptide is found in the brain, pituitary, heart, kidney, gastrointestinal tract, plasma (Roh *et al.*, 2004; Takei *et al.*, 2004a; Taylor *et al.* 2005; Takahashi *et al.*, 2006), pancreas, lung, spleen, thymus and ovary (Takei *et al.*, 2004b). Among these tissues, kidney, hypothalamus (Taylor *et al.*, 2005; Takahashi *et al.*, 2006) and stomach (Taylor *et al.*, 2005) have the highest peptide content. AM<sub>2</sub>/IMD was shown to be co-localized with vasopressin in the paraventricular (Takahashi *et al.*, 2006) and supraoptic nuclei of the human hypothalamus (Takahashi *et al.*, 2011). Under normal conditions, AM<sub>2</sub>/IMD is not found at a high level in the heart. AM<sub>2</sub>/IMD mRNA is detected in neonatal cardiomyocytes but is absent (Pan *et al.*, 2005) or sparse in adult myocardia in rat (Zhao *et al.*, 2006) and mouse (Zeng *et al.*, 2009). In the mouse, AM<sub>2</sub>/IMD is enriched in endothelial cells of coronary arteries and veins (Takei *et al.*, 2004b). However, this does not seem to apply to the adult human heart (Takahashi *et al.*, 2006).

## The sequence of AM<sub>2</sub>/IMD and its nomenclature

The amino acid sequences of various forms of AM<sub>2</sub>/IMD are shown in Figure 1. These are produced from a pre-pro hormone by cleavage between arginine residues at positions 93 and 94, to give a 53 amino acid version of AM<sub>2</sub>/IMD (Yang *et al.*, 2005). There is further potential for processing at the N-terminus to generate 40 and 47 amino acid versions of the peptide (Roh *et al.*, 2004). It is extremely important for researchers to specify which form they use in their studies. At this stage, it is difficult to ascertain which of these is the major peptide. Therefore, terms such as AM<sub>1-47</sub> and AM<sub>1-40</sub> are not correct if the N-terminally extended 53 amino acid is considered to be an active form of the peptide (Takahashi *et al.*, 2011). Another important nomenclature issue relates to the use of the terms 'AM<sub>2</sub>' or 'intermedin'. AM<sub>2</sub> is based on sound phylogenetic reasoning and allows ready accommodation of other members of the AM family such as AM<sub>5</sub>; however, 'intermedin' remains in common use. Intermedin was the former name for  $\alpha$ -melanocyte stimulating hormone. At the 7th International Symposium on the CGRP, Adrenomedullin, Amylin, Intermedin and Calcitonin (August 2010, Queenstown, New Zealand) the participants agreed that both terms should be used in the

abstract of any paper; throughout this review, AM<sub>2</sub>/IMD has been adopted. To differentiate between the forms of AM<sub>2</sub>/IMD, in this review, AM<sub>2</sub>/IMD-53, AM<sub>2</sub>/IMD-47 and AM<sub>2</sub>/IMD-40 are used.

## Known receptors for the CGRP/CT family of peptides

Given that AM<sub>2</sub>/IMD is a member of the CGRP/CT-family of peptides, it is logical to assume that it will produce at least some of its effects on their receptors. These are produced by the interaction of two GPCRs, the CT receptor and the calcitonin receptor-like receptor (CLR), with three receptor activity-modifying proteins (RAMPs). CLR with RAMP1 gives the CGRP receptor; with RAMP2 or RAMP3, it gives the AM<sub>1</sub> or AM<sub>2</sub> receptors. The nomenclature of the AM<sub>2</sub> receptor does not relate to the ability of the peptide AM<sub>2</sub>/IMD to activate this receptor. This is a potential source of confusion in the field. The CT receptor by itself is the receptor for CT; with the three RAMPs, it produces three amylin receptors: AMY<sub>1</sub>, AMY<sub>2</sub> and AMY<sub>3</sub>. The human CT receptor exists as two splice variants, distinguished as CT<sub>(a)</sub> and CT<sub>(b)</sub> receptors; in turn, this means that each amylin receptor exists as (a) or (b) forms (Poyner *et al.*, 2002). Specific information relating to the ability of AM<sub>2</sub>/IMD to act upon CT peptide family receptors is discussed later in this review.

## AM<sub>2</sub>/IMD antagonists

Very little work has been done to investigate the structure-activity relationship of AM<sub>2</sub>/IMD. It would be expected that it should follow the same themes as have been established for other members of this family, with agonist activation requiring the disulphide-bonded ring and subsequent truncation fragments acting as an antagonists, at least on stimulation of cAMP. AM<sub>2</sub>/IMD-47<sub>17-47</sub> is an antagonist (Roh *et al.*, 2004; Chauhan *et al.*, 2007), although in some systems, its inhibition appears to be non-competitive (Kandilci *et al.*, 2008), and it does not block all actions of AM<sub>2</sub>/IMD *in vivo* (White and Samson, 2007).

A number of novel antagonists have been produced by taking residues 22–30, 31–35 and 39–47 from AM<sub>2</sub>/IMD-47 and using them to replace the equivalent sections of AM<sub>22-52</sub>, the AM antagonist (Robinson *et al.*, 2009). The insertion of residues 23–30 unexpectedly gave a peptide that was a higher affinity antagonist than the parent compound at AM<sub>1</sub>, AM<sub>2</sub> and CGRP receptors. Residues 31–35 gave an antagonist that resembled the actions of AM<sub>22-52</sub> on AM<sub>1</sub> and CGRP receptors but which had a higher affinity on AM<sub>2</sub> receptors. Insertion of 39–47 gave an antagonist that resembled AM<sub>22-52</sub> at AM<sub>2</sub> and CGRP receptors but with a slightly lower affinity at AM<sub>1</sub> receptors. The ability of residues 22–30 to confer high affinity binding at CGRP receptors is interesting. In evolutionary terms, AM<sub>2</sub>/IMD is most closely related to AM with twice the number of identical residues compared to CGRP (Figure 1). However, in spite of this, at some receptors, it follows more closely CGRP potency than that of AM. It is possible that

**Table 1**

Distribution of AM2/IMD in mammalian tissues

Organs and tissues	Species	Techniques	References
<b>Cardiovascular system</b>			
Heart	Human	RT-PCR	Takahashi <i>et al.</i> (2006)
Left ventricles	Human	RIA	Morimoto <i>et al.</i> (2007)
Cardiomyocytes	Human	RIA	Morimoto <i>et al.</i> (2007)
Pericardial adipocytes	Human	RIA	Morimoto <i>et al.</i> (2007)
Vascular endothelial cells of pericardial veins	Human	RIA	Morimoto <i>et al.</i> (2007)
Vascular smooth muscle cells of coronary arteries	Human	ICC	Morimoto <i>et al.</i> (2007)
Myocardial cells	Human	ICC	Takahashi <i>et al.</i> (2006)
Heart	Rat	RIA	Taylor <i>et al.</i> (2005)
Endothelial cells of coronary vessels	Mouse	RT-PCR	Takei <i>et al.</i> (2004b)
<b>CNS</b>			
Brain	Teleost fish	RT-PCR	Ogoshi <i>et al.</i> (2003)
Brain	Human	RT-PCR	Takahashi <i>et al.</i> (2006)
Brain	Human	RIA, HPLC	Morimoto <i>et al.</i> (2007)
Paraventricular supraoptic nuclei of hypothalamus	Human	ICC	Takahashi <i>et al.</i> (2006)
Paraventricular and supraoptic nuclei of hypothalamus	Human	ICC	Morimoto <i>et al.</i> (2007)
Hypothalamus	Rat	RIA	Taylor <i>et al.</i> (2005)
Brainstem	Rat	RIA	Taylor <i>et al.</i> (2005)
Cerebellum	Rat	RIA	Taylor <i>et al.</i> (2005)
<b>Endocrine glands</b>			
Anterior and posterior lobes of pituitary	Human	ICC	Morimoto <i>et al.</i> (2007)
Pituitary	Human	RIA	Morimoto <i>et al.</i> (2007)
Pituitary	Human	RT-PCR	Takahashi <i>et al.</i> (2006)
Anterior pituitary			
Posterior pituitary	Rat	RIA	Taylor <i>et al.</i> (2005)
Pituitary	Rat	Northern blot	
Pituitary	Mouse	ICC	Roh <i>et al.</i> (2004)
Adrenal medulla	Human	RT-PCR, RIA, ICC	Morimoto <i>et al.</i> (2008)
<b>Excretory system</b>			
Kidney	Human	RT-PCR	Takahashi <i>et al.</i> (2006)
Kidney	Human	RIA, HPLC	Morimoto <i>et al.</i> (2007)
Renal tubular cells	Human	ICC	Morimoto <i>et al.</i> (2007)
Renal tubular cells	Human	ICC	Takahashi <i>et al.</i> (2006)
Renal arterioles	Human	ICC	Morimoto <i>et al.</i> (2007)
Kidney	Rat	RIA	Taylor <i>et al.</i> (2005)
Kidney	Mouse	RT-PCR	Takei <i>et al.</i> (2004b)
Glomeruli and vasa recta	Mouse	RT-PCR	Takei <i>et al.</i> (2004b)
<b>Gastrointestinal system</b>			
Gastrointestinal tract	Human	RT-PCR	Roh <i>et al.</i> (2004)
Stomach	Rat	RIA	Taylor <i>et al.</i> (2005)
Stomach	Rat	Northern blot	
Stomach	Mouse	ICC	Takei <i>et al.</i> (2004b)
Submaxillary gland, stomach, pancreas	Mouse	RT-PCR	Takei <i>et al.</i> (2004b)
<b>Reproductive system</b>			
Ovary	Mouse	PCR	Takei <i>et al.</i> (2004b)

Table 1

Continued

Organs and tissues	Species	Techniques	References
<b>Respiratory system</b>			
Lung	Rat	RIA	Taylor <i>et al.</i> (2005)
Pulmonary endothelium	Mouse	ICC, RT-PCR	Pfeil <i>et al.</i> (2009)
<b>Others</b>			
Lymphoid tissues	Mouse	RT-PCR	Takei <i>et al.</i> (2004b)
Plasma	Human	RIA, HPLC	Morimoto <i>et al.</i> (2007)
Plasma	Rat	RIA	Taylor <i>et al.</i> (2005)
Skin	Human	RT-PCR	Kindt <i>et al.</i> (2007)

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hAM2 / IMD-53      HSGPRRTQAQLLR-VGC-VLGTCCVQVQLSHRLWQLMGPPAGRQDSAPVDPSSPHSY
rAM2 / IMD-53      HVGSRRPHAQLLR-VGC-VLGTCCVQVQLSHRLWQLVRRPAGRRDSAPVDPSSPHSY
fAM2 / IMD1        HANNGGGRSHGQLMR-VAC-VLGTCCVQVQLSHRLYQLIGQSGKEDSSFMNPHSPHSY
hAM                YRQSMNFQGLRSFGC-RFGTCTVQKLAHQIYQFTD-KDKDNVAPRSKISPEQGY
hCGRP              AC-DTATCVTHRLAGLLSRSGGVVK-NNEVFVN-VGSKAF
hAmylin            KC-NTATCATQRLANFLVHSSNFC-AILSSTN-VGSNTY
hCT                CGNLSTCMLGTYTQDFNKF-----HTFFQTAIGVGAP

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Figure 1

The CT family of peptides. Sequences are human (h), rat (r), pufferfish (*Fugu rubripes*, f). Bold script on the AM2/IMD sequences indicates cleavage sites to produce 47 and 40 amino acid forms of the peptide. Shaded residues show identity compared to human AM2/IMD-53.

Table 2

Affinities of antagonists on CLR/RAMP receptors

Antagonist	CGRP	AM1	AM2	Reference
CGRP <sub>8-37</sub>	9–10	6–7	6–7	Poyner <i>et al.</i> (2002)
AM <sub>22-52</sub>	~6	7–8	7–8	
AM <sub>217-47</sub>	~6	~6	~6	Chauhan <i>et al.</i> (2007)

For CGRP<sub>8-37</sub> and AM<sub>22-52</sub>, pK<sub>i</sub> estimates are shown. For AM<sub>217-47</sub>, the value is the pA<sub>2</sub> estimated from inhibition of AM<sub>2</sub>/IMD-mediated relaxation of the rat mesenteric artery; the receptor responsible for this effect is unknown.

residues 22–30 are partly responsible for the CGRP-like properties of AM<sub>2</sub>/IMD. Equally, residues 31–35 may help give AM<sub>2</sub>/IMD its relatively high potency at AM<sub>2</sub> receptors, although the mechanistic basis for both of these observations remains obscure.

Whilst the studies with chimeric peptides may ultimately lead to better AM<sub>2</sub>/IMD antagonists, currently, AM<sub>2</sub>/IMD-47<sub>17-47</sub> is the only widely available compound, and it suffers from the problems of having at best a moderate affinity and limited selectivity. In this, it is not alone as none of the peptide antagonists at CGRP and AM receptors show particularly good discrimination between all of the receptors (Table 2). AM<sub>2</sub>/IMD-47<sub>17-47</sub> has not been tested against individual receptor subtypes and so its selectivity is unknown. The lack of a good AM<sub>2</sub>/IMD antagonist is a major barrier to

studying the physiological role of this peptide. Furthermore, identification of the cognate receptor for AM<sub>2</sub>/IMD is needed to develop a specific antagonist.

## The actions of AM<sub>2</sub>/IMD in mammals

### *The effective molecular forms of AM<sub>2</sub>/IMD*

Several forms of AM<sub>2</sub>/IMD have been tested in animals. Roh *et al.* (2004) observed that i.p. injection of AM<sub>2</sub>/IMD-47 is more potent than AM<sub>2</sub>/IMD-40 in increasing the heart rate. AM<sub>2</sub>/IMD-53 was found to elevate blood pressure and heart rate further than AM<sub>2</sub>/IMD-47 following an i.c.v. injection. On the other hand, AM<sub>2</sub>/IMD-47 produces more prominent hypotension than AM<sub>2</sub>/IMD-53 after i.v. injection (Ren *et al.*, 2006). Nevertheless, both of these peptides have been used to examine the pharmacological effects of AM<sub>2</sub>/IMD.

### *Cardiovascular effects of AM<sub>2</sub>/IMD*

Peripheral administration of AM<sub>2</sub>/IMD decreases aortic resistance, improves heart performance and elevates coronary perfusion flow under physiological conditions (Pan *et al.*, 2005; Dong *et al.*, 2006). This peptide also strengthens the contractility of the left ventricle and increases the coronary perfusion flow and heart beats following ischemia/reperfusion injury (Yang *et al.*, 2005). AM<sub>2</sub>/IMD has been shown to reduce the myocardial infarction size (Song *et al.*, 2009; Zeng *et al.*, 2009), hypertrophy in left ventricular car-

diomyocytes and the production of cardiac fibrosis (Yang *et al.*, 2009) under pathological conditions. On the other hand, AM2/IMD decreases arterial pressure (Chang *et al.*, 2004; Roh *et al.*, 2004; Takei *et al.*, 2004b; Pan *et al.*, 2005; Fujisawa *et al.*, 2006; Abdelrahman and Pang, 2007). The hypotensive effect of AM2/IMD is attributed to its vasodilatory action as it does not alter mean circulatory filling pressure (Abdelrahman and Pang, 2006). This peptide essentially dilates the blood vessels in the renal (Fujisawa *et al.*, 2004; 2006; Takei *et al.*, 2004b), pulmonary (Burak Kandilci *et al.*, 2006) and abdominal arteries (Jolly *et al.*, 2009), improving blood flow in the visceral organs. However, central administration of AM2/IMD increases sympathetic activity (Hashimoto *et al.*, 2007; Takahashi *et al.*, 2011) and elevates blood pressure (Ren *et al.*, 2006; Hashimoto *et al.*, 2007; Takahashi *et al.*, 2011). These results suggest that AM2/IMD is potentially an important regulatory factor in cardiovascular activity via central and peripheral actions. Furthermore, the level of AM2/IMD mRNA and protein is enhanced in the myocardium (Pan *et al.*, 2005; Zeng *et al.*, 2009), aorta and plasma (Zeng *et al.*, 2009) in cardiac hypertrophy or heart infarction. It is an interesting possibility that the increased AM2/IMD may stimulate sensitive sympathetic visceral afferents to evoke reflex excitation of the cardiovascular system inducing angina and the referred pain.

### Other effects of AM2/IMD

AM2/IMD may play a role in the regulation of prolactin release during reproduction in females. Both central (Taylor and Samson, 2005) and peripheral (Lin Chang *et al.*, 2005) injections of AM2/IMD have been shown to increase blood prolactin levels. This peptide inhibits growth hormone-releasing hormone (GHRH)-stimulated growth hormone release from anterior pituitary (Taylor *et al.*, 2006) and elevates the levels of oxytocin (Hashimoto *et al.*, 2005; 2007; Taylor and Samson, 2005) and vasopressin in plasma (Taylor and Samson, 2005). In addition, AM2/IMD was found to induce anti-diuresis and anti-natriuresis (Takei *et al.*, 2004b), decrease food (Roh *et al.*, 2004; Taylor *et al.*, 2005) and water (Taylor *et al.*, 2005) intake and inhibit gastric emptying activity (Roh *et al.*, 2004). These studies suggest that AM2/IMD produces multiple actions and may be involved in controlling the homeostasis of growth and metabolism.

### Comparison of the effects of AM2/IMD and AM

Many studies have reported that AM2/IMD is more potent than AM in both periphery and brain. Furthermore, AM2/IMD and AM may not always display identical effects in some physiological conditions. For example, AM2/IMD inhibits GHRH-stimulated release of growth hormone from rat anterior pituitary cell cultures, whereas AM does not (Taylor *et al.*, 2006). Down-regulation of AM2/IMD expression was maintained in the remnant kidney of 5/6 nephrectomized rats (a model of chronic renal impairment), but AM expression was elevated in the later phase in these kidney tissues, suggesting that AM2/IMD has a distinct pathophysiological role.

## The pharmacology of AM2/IMD on molecularly defined receptors

### Introduction

AM2/IMD has been examined on CGRP, AM and AMY receptors, either on transfected cells (Chang *et al.*, 2004; Roh *et al.*, 2004; Takei *et al.*, 2004a; Hay *et al.*, 2005; Qi *et al.*, 2008; 2011; Wunder *et al.*, 2008) or on CGRP receptors endogenously expressed in human SK-N-MC and rat L6 cells (Chang *et al.*, 2004; Roh *et al.*, 2004). The results of these studies are summarized in Table 3.

An important issue to consider is whether the form of AM2/IMD makes any difference to its pharmacology because some differences in the biological activities of AM2/IMD-47 and AM2/IMD-40 have been reported, although their overall behaviour is broadly similar (Pan *et al.*, 2005). In a direct comparison of AM2/IMD-47 and AM2/IMD-40 on the CGRP receptor of rat L6 cells, AM2/IMD-40 was more potent, although the difference was less clear on the CGRP receptor in human SK-N-MC cells. Furthermore, in binding experiments carried out on the two cell lines, there was little difference in their  $K_i$  values (Roh *et al.*, 2004), although as noted below, the interpretation of these is not simple. The two forms have also been examined on Cos 7 cells transiently transfected with human CGRP and AM<sub>2</sub> receptors, although not in parallel studies (Hay *et al.*, 2005; Qi *et al.*, 2008). There was little difference between their potencies, but given the variation in potency seen in transient transfections, little can be drawn from this. However, if there were differences in the potency between the two forms, this should have been reflected in their relative potency compared with CGRP. On both receptors, the potency relative to CGRP was very similar. At present, it seems that there is not a compelling case to assume that there are meaningful differences in the potencies of the two AM2/IMD forms at human receptors, at least when measuring cAMP, although further work is needed.

### The receptor selectivity of AM2/IMD in functional studies

The data in Table 3 suggest that AM2/IMD shows selectivity between different receptor types. This has been re-plotted in Figure 2, to compare the potency of AM2/IMD on the AM<sub>1</sub> and AM<sub>2</sub> receptors relative to that for the CGRP receptor, or the AMY<sub>2</sub> and AMY<sub>3(a)</sub> receptors relative to that for the AMY<sub>1(a)</sub> receptor. For the purposes of this table, no distinction has been made between the forms of AM2/IMD. It appears that AM2/IMD shows selectivity for the AM<sub>2</sub> receptor, being around an order of magnitude more potent at that receptor than either the CGRP or AM<sub>1</sub> receptors. Nevertheless, it is important to point out that due to the lack of quantification in some of the published studies, we can only estimate potencies in some cases. There are less data for the CT receptor-based receptors, but what there is suggests a potency order of AMY<sub>1(a)</sub>  $\geq$  AMY<sub>3(a)</sub>  $\gg$  AMY<sub>2</sub> = CT<sub>(a)</sub>. For the AMY<sub>2</sub> receptor, there are only two studies that were performed in Cos 7 cells, where this receptor usually forms only poorly (Christopoulos *et al.*, 1999; Hay *et al.*, 2005), and there is insufficient evidence in the report to assess whether there was any formation of this entity or whether the AM2/IMD was simply acting on a CT receptor (Takei *et al.*, 2004a).

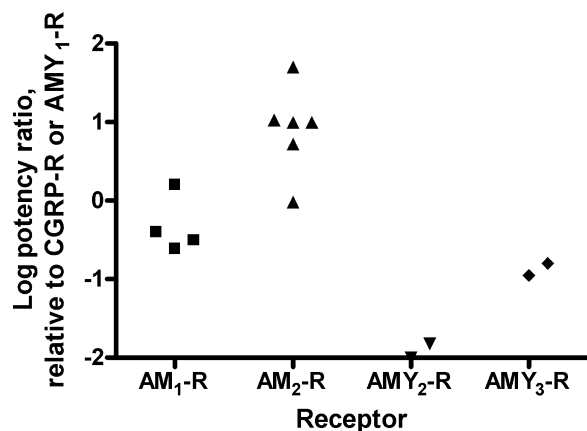


**Table 3**

pEC<sub>50</sub> values or estimates for AM2/IMD and other peptides at CGRP, CT, AM and amylin (AMY) receptors

Cell type	Ligand	CGRP receptor	AM <sub>1</sub> receptor	AM <sub>2</sub> receptor	CT receptor	AMY <sub>1(a)</sub> receptor	AMY <sub>2</sub> receptor	AMY <sub>3(a)</sub> receptor	Reference
Cos 7	AM2/IMD-47	8.24 ± 0.12		8.96 ± 0.17					Qi <i>et al.</i> (2011)
	AM	8.04 ± 0.15		9.76 ± 0.18					
	CGRP	9.84 ± 0.12		6.77 ± 0.17					
Cos 7	AM2/IMD-40	8.71 ± 0.13	8.10 ± 0.04	8.69 ± 0.13	6.53 ± 0.45	8.07 ± 0.19	6.25 ± 0.26	7.12 ± 0.19	Hay <i>et al.</i> (2005)
	hAM				6.73 ± 0.45	6.48 ± 0.28		6.89 ± 0.51	
	hCGRP	9.47 ± 0.19	6.39 ± 0.10	6.87 ± 0.13	6.80 ± 0.05	8.70 ± 0.17	7.24 ± 0.19	7.60 ± 0.17	
	hCT				8.99 ± 0.10	8.93 ± 0.09		8.02 ± 6.22	
Cos 7	AM2/IMD-47	9.07 ± 0.07		10.1 ± 0.02					Qi <i>et al.</i> (2008)
	AM	8.27 ± 0.11		9.92 ± 0.06					
	CGRP	10.10 ± 0.11		7.36 ± 0.06					
HEK-293T	AM2/IMD-40	7.5 ?	7.7 ?	9.2					Roh <i>et al.</i> (2004)
	AM	8	8.8	8					
	CGRP	9.5	7.7?	8					
	AM2/IMD-40	8.2?							
L6	AM2/IMD-47	7.6?							
	CGRP	9.2							
	AM	8							
	AM2/IMD-40	8.6							
SK-N-MC	AM2/IMD-40	7 ?							
	AM2/IMD-47	9.3							
	CGRP	7.7							
	AM	7.9							
Cos 7	AM2/IMD	7.9	7.5	8.9	6?	8	6?	7.2	Takei <i>et al.</i> (2004)
	AM	8	9.9	9.7					
	CGRP	9.8	7.5?	7.5					
	hCT				9	9	9.2	7.8	
HEK-293T	AM2/IMD	8	7.5	9				Chang <i>et al.</i> (2004)	
CHO	AM2/IMD	7.74 ± 0.09	8.27 ± 0.06*						Wunder <i>et al.</i> (2008)
	AM	8.44 ± 0.03	9.45 ± 0.08						
	AM	8.44 ± 0.03	9.45 ± 0.08						
	CGRP	9.50 ± 0.12	6.87 ± 0.08						

Where the concentration–response curves are incomplete, a question mark has been placed next to the pEC<sub>50</sub>. \*Partial agonist. Values without error bars have been estimated from published concentration–response curves.



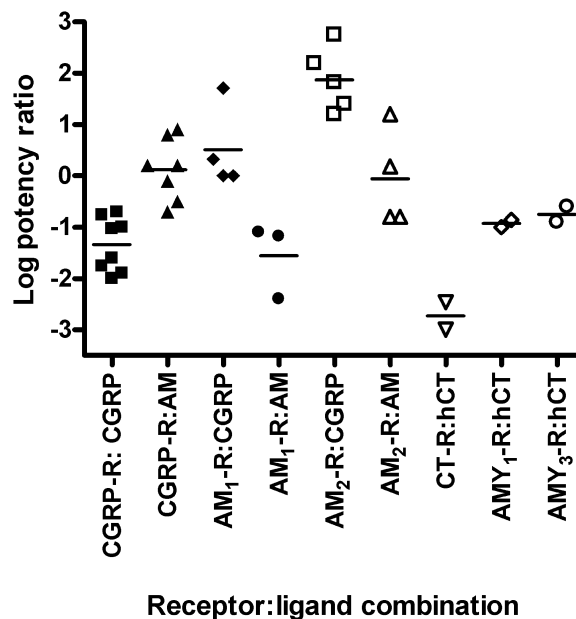
**Figure 2**

Potency ratios of AM2/IMD at CLR and CTR-based receptors. Log potency ratios were calculated from the data in Table 1. Points show the results of individual studies as indicated in Table 1. For the AM<sub>1</sub> and AM<sub>2</sub> receptors, the EC<sub>50</sub> of AM2/IMD was compared with that at the CGRP receptor. For the AMY<sub>2</sub> and AMY<sub>3</sub> receptors, the EC<sub>50</sub> of AM2/IMD was compared with that at the AMY<sub>1</sub> receptor. No distinction has been made between AM2/IMD-40 and AM2/IMD-47; data from rat L6 have also been included with the human forms of the CGRP receptor.

The relative potency of AM2/IMD compared to CGRP and AM depends on the receptor type (Table 2, Figure 3). At the CGRP receptor, AM2/IMD is at least an order of magnitude less potent than CGRP itself but is roughly equipotent with AM. At the AM<sub>1</sub> receptor, it is typically a little less potent than CGRP but is two or more orders of magnitude less potent than AM. At the AM<sub>2</sub> receptor, it is between 10- and 100-fold more potent than CGRP and is comparable with AM itself. There is again less data for amylin and CT receptors. Along with AM and CGRP, AM2/IMD has a very low potency at the CT receptor compared with CT itself. At the AMY<sub>1(a)</sub> and AMY<sub>3(a)</sub> receptors, it is around an order of magnitude less potent than CT. Taking the data as a whole, arguably the most interesting observation is that at the AM<sub>2</sub> receptor, the potency of AM2/IMD approaches the potency of AM itself, the presumed endogenous ligand. It seems possible that this receptor may function physiologically as an AM2/IMD receptor. This is supported by recent data in rat and mouse AM<sub>2</sub> receptors, where AM and AM2/IMD-47 are equipotent (DLH, unpubl. obs.).

### The receptor selectivity of AM2/IMD in radioligand binding studies

Agonist potency ratios are an unsatisfactory way of investigating affinity of ligands at receptors as they reflect both binding and efficacy; the latter is very system dependent. Unfortunately, very few radioligand binding studies have been carried out with AM2/IMD. Nevertheless, data on AMY<sub>1(a)</sub> and AMY<sub>3(a)</sub> receptors confirm that the potency ratios seen at stimulating cAMP production are in line with affinities (Table 4) (Hay *et al.*, 2005). For CGRP receptors, the situation is less clear cut. AM2/IMD has been investigated on rat L6 and human SK-N-MC cells using iodinated CGRP as the



**Figure 3**

Agonist potency ratios for AM2/IMD on CGRP, AM and amylin receptors. Log potency ratios are defined as  $\log [EC_{50} \text{ AM2/IMD} \div EC_{50} \text{ agonist}]$  and were calculated from the data in Table 1. Points show the results of individual studies as indicated in Table 1. For the CGRP and AM receptors, potency ratios have been calculated for CGRP and AM; for the CT and amylin receptors, the potency ratio for human calcitonin has been calculated. No distinction has been made between AM2/IMD-40 and AM2/IMD-47; data from rat L6 have also been included for the CGRP receptor.

**Table 4**

Affinities of AM2/IMD and related peptides at AMY receptors

Ligand	hAMY <sub>1(a)</sub> receptor	hAMY <sub>3(a)</sub> receptor
hCT	6	≤6
rAMY	8.76 ± 0.06	8.60 ± 0.09
hαCGRP	8.00 ± 0.08	6.97 ± 0.55
hAM	<6	<6
AM2/IMD-40	6.93 ± 0.69	6.21 ± 0.26

Receptors were expressed in Cos 7 values. The table shows pIC<sub>50</sub> values for inhibition of [<sup>125</sup>I]-rAMY binding on whole cells. The values are mean ± SEM for three independent experiments (Hay *et al.*, 2005).

radioligand (Roh *et al.*, 2004). This should measure binding just to CGRP receptors. However, in neither case does the binding profile of the receptors match what would be expected for a CGRP receptor, as there is little difference between the IC<sub>50</sub> values for CGRP or AM. On L6 cells, both AM2/IMD-47 and AM2/IMD-40 have around a fivefold lower affinity than AM or CGRP; on SK-N-MC cells the differences appears less. Given the poor discrimination between CGRP and AM, it is difficult to know how to interpret these results.

Table 5

Studies of pharmacology of AM2/IMD *in vivo* and in animal tissues

AM2/IMD-induced response	Peptide	Species	Antagonism			References
			CGRP <sub>8-37</sub>	AM <sub>22-52</sub>	AM <sub>217-47</sub>	
Reduction of arterial pressure	AM2/IMD-47, i.p.	Rat	+		+	Roh <i>et al.</i> (2004)
	AM2/IMD-47, i.p.	SHR	+	-		
Increase of arterial pressure	AM2/IMD-47, i.c.v.	Rat	Partially			Taylor <i>et al.</i> (2005)
Increase of heart rate			Partially			
Reduction of arterial pressure	AM2/IMD-53, i.c.v.	Rat	+	+		Ren <i>et al.</i> (2006)
Vasodilation of pulmonary artery	AM2/IMD-47, i.a.	Rat	+			Burak Kandilci <i>et al.</i> (2006)
Increase of heart rate	AM2/IMD-47, i.c.v.	Rat		-		
Increase in plasma oxytocin			Partially	Partially		Hashimoto <i>et al.</i> (2007)
c-fos gene expression in the supraoptic and paraventricular nuclei			Partially	Partially		
cAMP generation in spinal cord	AM2/IMD-47, p.f.	Rat	Partially	Partially		Owji <i>et al.</i> (2008)
Increase of heart rate	AM2/IMD-53, i.v.	Rat		-		Jolly <i>et al.</i> (2009)
Reduction of arterial pressure				-		
Vasodilatation						
Abdominal aorta				-		
Renal mesenteric artery			+			
cAMP generation in myocardium and aorta	AM2/IMD-53, p.f.	SHR	-	-		Zeng <i>et al.</i> (2009)
Reduction of permeability of lung blood microvascular endothelial cells	AM2/IMD-47, p.f.	Human	+			Pfeil <i>et al.</i> (2009)

p.f., perfusion; SHR, spontaneous hypertensive rat. +, antagonism; -, no antagonism; partially, partially antagonism; blank, not tested.

### The pharmacology of AM2/IMD at fish receptors

One study has examined the pharmacology of AM2/IMD at puffer fish, *Takifugu obscurus*, receptors (Nag *et al.*, 2006). This expresses orthologues of mammalian RAMP1, RAMP2 and RAMP3 (RAMP2 exists in two splice forms) as well as two extra RAMPs. This species also has three CLR orthologues. *Fugu* AM2/IMD showed almost 100-fold selectivity for CLR1/RAMP3 compared with the other RAMPs, although its potency at this receptor was little different from the AM1 or AM5 peptides. It was also a potent agonist at the CLR3/RAMP3 receptor. Here it was more potent than the AM5 peptide; there was little difference in potency compared to the AM1 peptide, but it may have had greater efficacy. Given the evidence that AM2/IMD appears to preferentially activate CLR/RAMP3 (the AM<sub>2</sub> receptor) in humans, it is interesting this RAMP is also involved in its recognition in fish.

### Conclusions

Overall, a number of themes are apparent from considering the pharmacology of AM2/IMD on defined receptors. There is clearly a need for further work, to properly define the specificity of these agents. In particular, there is a need for radioligand binding studies to allow pK<sub>i</sub> values to be measured. That said, the peptide does seem to show an interesting pharmacological profile and appears to have good activity at the AM<sub>2</sub> receptor but with lower potency at the

AM<sub>1</sub> receptor. There is no known receptor where it is more potent than the likely endogenous ligand. The role of the N-terminal extensions of AM2/IMD is unclear. Whilst there is a solid body of work to indicate that these can influence the potency of the peptides, it is uncertain as to whether they do this by actions at the level of the receptors or whether they influence factors such as susceptibility to peptidases. This is particularly the case when considering the actions of these peptides in *in vivo* studies.

### The receptor pharmacology of AM2/IMD in native cells and tissues

The data reviewed above clearly show that in recombinant systems, AM2/IMD can activate CGRP, AM<sub>1</sub> and AM<sub>2</sub> receptors. On moving into endogenous cells and tissues, it is important to determine which of these three receptors chiefly mediates the effects of the peptide, whether exogenously applied or released naturally and also to ascertain if there are any other receptors for these peptides. The data regarding receptors mediating AM2/IMD-induced pharmacological responses in native tissues and cells are variable.

The CGRP receptor antagonist CGRP<sub>8-37</sub> (Chiba *et al.*, 1989) and AM receptor antagonist AM<sub>22-52</sub> (Hay *et al.*, 2003) have been used to determine if AM2/IMD-induced responses are independent of CGRP or AM receptors (Table 5). Roh *et al.* (2004) reported that AM2/IMD-induced hypotension was

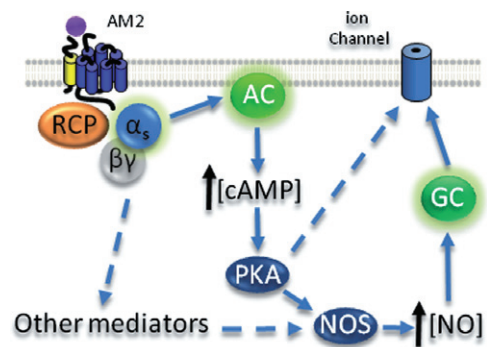


blocked by CGRP<sub>8-37</sub>. Other studies show that AM<sub>22-52</sub> or/and CGRP<sub>8-37</sub> was equally effective in attenuating the effects induced by AM2/IMD and AM. These include peripheral vasodilatation in the renal and mesenteric arteries (Jolly *et al.*, 2009), central increase in blood pressure and heart rate (Ren *et al.*, 2006) and barrier-protective effect of AM2/IMD on pulmonary endothelial cells (Pfeil *et al.*, 2009). These results suggest that CGRP or AM receptors mediate AM2/IMD-induced responses.

However, other studies have demonstrated that AM2/IMD may activate a distinct receptor besides the CGRP and AM receptors in native cells and tissues. Our (YH, RQ) laboratory characterized pharmacological response to AM2/IMD using dispersed rat embryonic spinal cultured cells. We observed that AM2/IMD-47 competed for specific [<sup>125</sup>I]-AM binding in a biphasic manner with pIC<sub>50</sub>s of 9.03 ± 0.22 and 6.45 ± 0.24 respectively. The fraction of high affinity site was 41 ± 5.0% of the total sites. However, AM followed a one component competition model (pIC<sub>50</sub> = 9.49 ± 0.17) under the same experimental conditions. These findings suggest that [<sup>125</sup>I]-AM binds to two different classes of sites or two affinity states of a single entity distinguishable by AM2/IMD-47 but not unlabelled AM. AM2/IMD-47 also demonstrated high affinity for specific [<sup>125</sup>I]-CGRP binding with a pIC<sub>50</sub> (9.73 ± 0.06) close to rat αCGRP (9.99 ± 0.16), indicating that AM2/IMD-47 displays similar affinity for specific [<sup>125</sup>I]-CGRP binding to CGRP itself (Owji *et al.*, 2008). These results suggest that AM2/IMD can recognize CGRP as well as AM receptors. Our observations are consistent with cell line data. However, we have further demonstrated that the AM2/IMD-47-induced increase in cAMP production in rat spinal cord cells is only partially antagonised by BIBN4096BS (non-peptide CGRP antagonist), hAM<sub>22-52</sub> and CGRP<sub>8-37</sub> individually. Even a combination of BIBN4096BS, hAM<sub>22-52</sub> and the amylin receptor antagonist AC187 (Owji *et al.*, 2008) did not fully inhibit the response, indicating that AM2/IMD could act at a distinct receptor besides CGRP, AM and AMY receptors. These results are supported by a few *in vivo* studies. It has been shown that AM2/IMD-induced activation of *c-fos* gene in the supraoptic and the paraventricular nuclei of hypothalamus and increased plasma oxytocin (Hashimoto *et al.*, 2007) and cAMP generation in the myocardium and aorta (Zeng *et al.*, 2009) can only be partially blocked by CGRP<sub>8-37</sub> and AM<sub>22-52</sub>. On the other hand, the antagonists tested have only low affinity at rat AM receptors (Hay *et al.*, 2002; 2003). Therefore, it is also possible that AM receptors make some contribution to the responses. Evidently, more work is needed to study the receptors mediating the actions of AM2/IMD.

## Signalling pathways

CLR belongs to the super-family of seven transmembrane GPCRs (Mittra and Bourreau, 2006). It undergoes conformational changes resulting in coupling to G<sub>s</sub>, activation of adenylyl cyclase and accumulation of intracellular cAMP when activated by an agonist (Shimekake *et al.*, 1995). If AM2/IMD acts through CLR, it is also logical to assume that it will activate these signalling pathways. The pharmacological effects of AM2/IMD are believed to be attributed to the activation of cAMP/protein kinase A signalling pathway, but



**Figure 4**

Possible signalling pathways activated by AM2/IMD in vascular and endothelial tissue (Kandilci *et al.*, 2008; Grossini *et al.*, 2008). Presumably via CLR, AM2/IMD activates G<sub>αs</sub> leading to modulation of ion channel activity via protein kinase A (PKA)-dependent or independent mechanisms. Broken arrows represent pathways that are incompletely understood. Note that for the purposes of this diagram, additional components of some pathways are not shown. AC, adenylyl cyclase, GC, guanylate cyclase, RCP, receptor component protein.

other pathways may also play a role (Figure 4). Perfusion with AM2/IMD in the heart increases cAMP content in the myocardium or aorta (Pan *et al.*, 2005; Yang *et al.*, 2005; Zeng *et al.*, 2009), and the protein kinase A inhibitor H-89 abolishes AM2/IMD-induced inotropic effect (Dong *et al.*, 2006). It has also been demonstrated that the activation of protein kinase C (Dong *et al.*, 2006) and Akt/Glycogen synthase kinase-3β signalling pathways (Song *et al.*, 2009) contributes to the cardiovascular action of AM2/IMD. In addition, the NO signalling pathway mediates some AM2/IMD-induced effects. This peptide was shown to increase NO content and NOS activity in isolated rat aortas (Yang *et al.*, 2006). Treatment with the NOS inhibitor L-NAME reduces AM2/IMD-induced renal vasodilator response (Jolly *et al.*, 2009). Where it has been examined, AM2/IMD produces vasodilation in an endothelial-dependent manner, by production of NO, activation of guanylate cyclase and opening of calcium-dependent large-conductance K<sup>+</sup> channels (Kandilci *et al.*, 2008) (Figure 4). However, since both AM and CGRP can also produce endothelial-independent vasodilation in the appropriate vascular beds, it is to be anticipated that AM2/IMD will also act in this way.

## Conclusion: the nature of the receptors for AM2/IMD

The fact that AM2/IMD displays overlapping distribution and shares certain similarities with AM implies that it has similar bioactivities. In the absence of selective antagonists, most studies have relied on addition of exogenous AM2/IMD to explore its potential role; this approach has obvious limitations. The physiological relevance of AM2/IMD and in particular the role it may play distinct from that of AM warrants further studies but these are likely to need additional pharmacological tools.

An important issue for ongoing AM2/IMD studies is that its receptors are not yet clearly identified. There is no doubt that AM2/IMD can act pharmacologically through the existing CGRP and AM receptors as well as AMY<sub>1</sub> and AMY<sub>3</sub> receptors. Of these known receptors, it seems to have its highest affinity at the AM<sub>2</sub> receptor where some studies have suggested it may be equipotent with AM. This is worth considering as a candidate for a receptor through which AM2/IMD exerts its physiological effects, but further work is needed to investigate this.

There are also suggestions that AM2/IMD might activate receptors that are distinct from the CLR and CTR complexes, or are modified by additional accessory proteins. There are examples in intact tissues and *in vivo* where AM2/IMD appears more potent than AM or CGRP, a situation that cannot be easily reconciled with the profile of these peptides on cloned receptors. Here considerations such as the relative metabolic stability of AM2/IMD compared with AM and CGRP may influence potency, although this has been little explored. Some effects also are resistant to a combination of CGRP, AMY and AM receptor antagonists. No work has been done to explore whether AM2/IMD shows a different pattern of coupling to second messenger pathways compared with CGRP, AM or AMY; if this happens, it will further complicate the analysis of its actions. Therefore, further research is needed to identify the receptors that mediate the actions of AM2/IMD.

## Acknowledgements

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## Conflicts of interest

None to declare.

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