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REVIEW ARTICLE

Amylin structure–function relationships and receptor pharmacology: implications for amylin mimetic drug development

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Amylin is an important, but poorly understood, 37 amino acid glucoregulatory hormone with great potential to target metabolic diseases. A working example that the amylin system is one worth developing is the FDA-approved drug used in insulin-requiring diabetic patients, pramlintide. However, certain characteristics of pramlintide pharmacokinetics and formulation leave considerable room for further development of amylin-mimetic compounds. Given that amylin-mimetic drug design and development is an active area of research, surprisingly little is known about the structure/function relationships of amylin. This is largely due to the unfavourable aggregative and solubility properties of the native peptide sequence, which are further complicated by the composition of amylin receptors. These are complexes of the calcitonin receptor with receptor activity-modifying proteins. This review explores what is known of the structure–function relationships of amylin and provides insights that can be drawn from the closely related peptide, CGRP. We also describe how this information is aiding the development of more potent and stable amylin mimetics, including peptide hybrids.

Abbreviations

AM, adrenomedullin; AMY, amylin receptor; CLR, calcitonin receptor-like receptor; CT, calcitonin; ECD, extracellular domain; IAPP, islet amyloid polypeptide; RAMP, receptor activity-modifying protein

Tables of Links

TARGETS	
GPCRs	
AMY_1 receptor	GLP-1 receptor
AMY_2 receptor	RAMP1
AMY_3 receptor	RAMP2
CTR, CT receptor	RAMP3

LIGANDS	
AC187	β-CGRP
AM, adrenomedullin	$CGRP_{8–37}$
AM2, intermedin	CT, calcitonin
Amylin	GLP-1
α-CGRP	Pramlintide

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

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Introduction to amylin and the calcitonin family of peptides

Amylin is a centrally acting, neuroendocrine hormone synthesized with insulin in the beta cells of pancreatic islets. Co-secretion is provoked by nutrient influx to the gastrointestinal tract, signalling the need to restore blood-glucose homeostasis. Insulin triggers glucose uptake in muscle and liver cells, effectively removing glucose from the bloodstream and making it available for energy use and storage. Amylin regulates glucose homeostasis by inhibiting gastric emptying, inhibiting the release of the counter-regulatory hormone glucagon and inducing meal-ending satiety (Hay *et al.*, 2015).

Human amylin was probably first observed as early as 1901, described as hyaline deposits found in the pancreatic islets of patients with type 2 diabetes (Opie, 1901). Amylin was later characterized as an amyloidogenic peptide, isolated from a beta cell tumour and called islet amyloid polypeptide (IAPP), and then, amylin (Westermark *et al.*, 1986). Physiologically, amylin functions as a glucoregulatory and satiety-inducing hormone, which is protective against postprandial spikes in blood glucose and overeating (see Hay *et al.*, 2015; Hinshaw *et al.*, 2016). Under disease conditions, amylin becomes dysregulated, misfolds, self-associates and forms amyloid deposits (see Akter *et al.*, 2016), but the role of amylin in disease pathogenesis remains unclear.

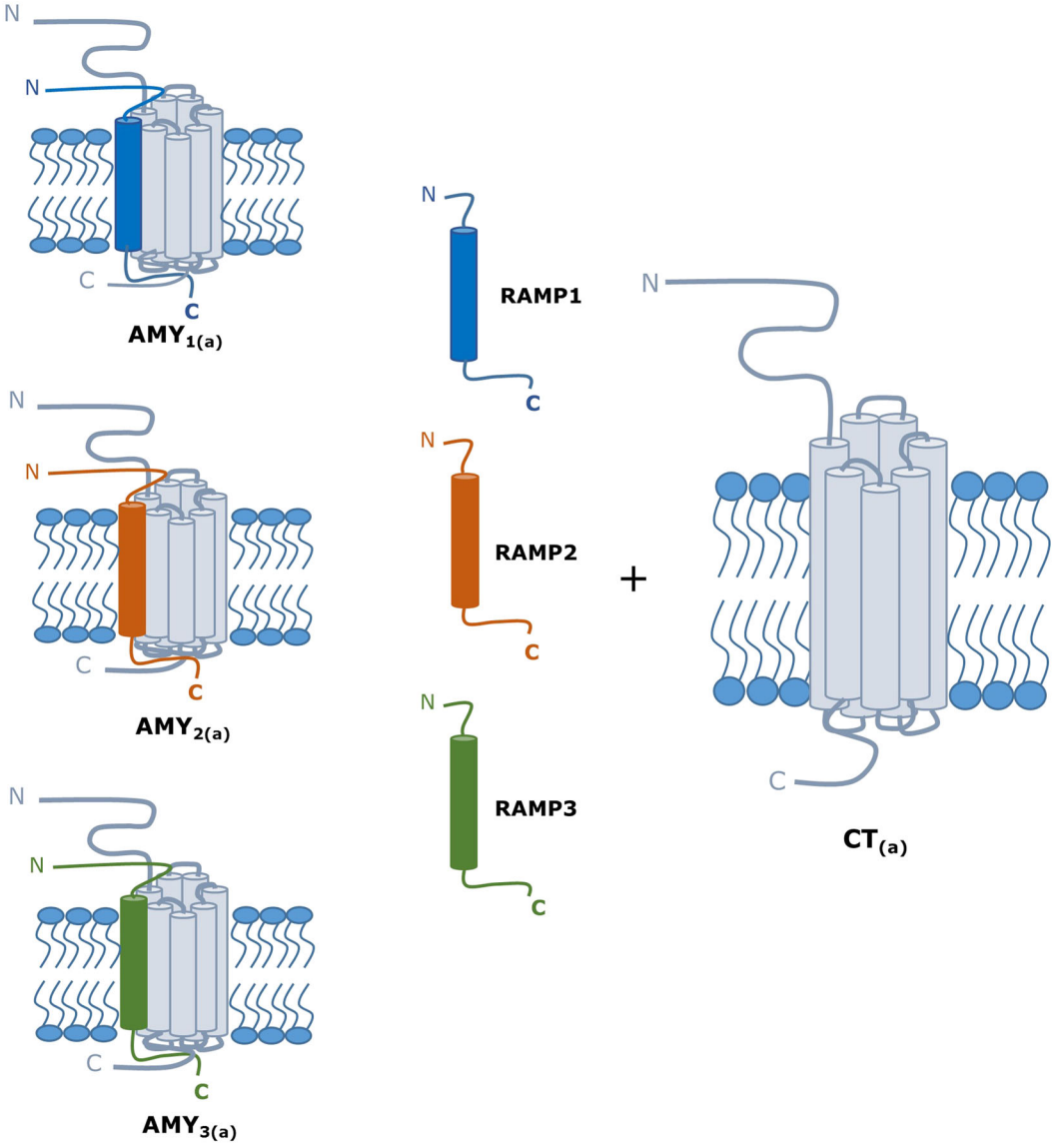

Figure 1

Amylin receptor components comprise a family B GPCR, the calcitonin receptor. Depicted here is the more common splice variant without the 16 amino acid insert in intracellular loop 1, $CT_{(a)}$. This core GPCR interacts with one of three accessory proteins (RAMPs), which alter the pharmacology and downstream signalling of the receptor.

Amylin is a member of the calcitonin (CT) family of peptides, which includes CT itself, the CGRPs comprising two variants (αCGRP and βCGRP), adrenomedullin (AM) and AM2 (intermedin). All members of this family are clinically relevant drug targets due to their roles in the regulation of several critical homeostatic processes (Hay and Dickerson, 2010).

In the case of amylin, its beneficial physiological effects on postprandial blood-glucose and meal-ending satiation have made it a suitable target in diabetes, validated by the FDA approval of the amylin analogue, pramlintide for insulin-requiring diabetes (Schmitz *et al.*, 1997). Despite this, the pharmacokinetic profile of pramlintide and its formulation requirements make it a suboptimal drug (Weyer *et al.*, 2001). In particular, the additional beneficial effects of amylin or pramlintide in reducing body weight and their synergistic actions with other metabolic hormones are unlikely to translate into drugs for obesity without further improvement to the molecule or formulation. Therefore, there is considerable scope to improve upon amylin and existing amylin mimetics to optimize their therapeutic potential. Insight into how this may be achieved requires unlocking the mechanisms of amylin peptide binding and activation of its receptor(s), and hence, how the amino acid sequence and structure of this peptide translates into function.

Amylin receptors

Amylin and other CT family peptides are ligands for family B GPCRs. The peptides range from 32 to 52 amino acids in length, and they activate GPCRs, which can heterodimerize with accessory proteins called receptor activity-modifying proteins (RAMPs). Three RAMP genes are expressed in humans, encoding RAMP1, RAMP2 and RAMP3, with 31% sequence identity between them (McLatchie *et al.*, 1998). GPCR association with RAMPs adds an intriguing layer of complexity to receptor activity because RAMPs can change the pharmacology, trafficking, degradation/recycling pathways, glycosylation state, and/or downstream signalling of associated GPCRs (see Hay and Pioszak, 2016).

Amylin receptors comprise a core family B GPCR, the CT receptor (CTR), associated with the three RAMPs (Figure 1). Encoded on chromosome 12 in humans, CTR has two major splice variants, $hCT_{(a)}$ and $hCT_{(b)}$, of which the former is the major subtype. $hCT_{(b)}$ has a 16 amino acid insert in its first intracellular loop (Gorn *et al.*, 1992). Cloning from MCF-7 and BIN-67 cells showed that residue 447 in $hCT_{(a)}$ and $hCT_{(b)}$ is a proline but is a leucine in $hCT_{(a)}$ cloned from T47D cells. Another human variant was described from MCF-7 cells which, like $hCT_{(a)}$, not only lacks the 16 amino acids in the first loop but is also lacking 47 amino acids of its N-terminal extracellular domain (ECD) (Albrandt *et al.*, 1995). In rats, $rCT_{(a)}$ is equivalent to $hCT_{(a)}$, and there is also a splice variant, rC1b, which has an additional 37 amino acids in the second extracellular loop (Poyner *et al.*, 2002).

The two main CTR isoforms, combined with the three RAMPs, yield six amylin receptors. The physiological relevance of each of the possible amylin receptor subtypes is not well understood, but a range of studies have described the pharmacological properties of many of these receptors (Poyner *et al.*, 2002; Hay *et al.*, 2015).

Amylin receptor pharmacology

Association of CTR with RAMPs confers an increase in amylin affinity, compared with CTR alone, and an increase in potency in functional assays, so-called induction of amylin receptor phenotype. Splice variants, RAMP association, cell type in which the receptor is expressed (and thus background signalling protein expression) and sequence differences between species lead to considerable complexity in pharmacology (Tilakaratne *et al.*, 2000; Udawela *et al.*, 2006a, b; Morfis *et al.*, 2008). Despite this complexity, patterns of receptor pharmacology have emerged, and these are summarized in the Guide to Pharmacology (currently only for human receptors; www.guidetopharmacology.org). In particular, readers should refer to the most recent edition of the Concise Guide to Pharmacology to obtain the up-to-date rank orders of potency of agonists and antagonists (Alexander *et al.*, 2015). The general consensus for agonist pharmacology is that amylin is a high-affinity/potency ligand of AMY_1 and AMY_3 receptors and variably of AMY_2 receptors; binding studies are generally used for the latter receptor. $AMY_{1(a)}$ and $AMY_{3(a)}$ receptors are the most extensively characterized and show variable responsiveness to CGRP, depending on the RAMP and species. The pharmacology of agonists at human CT and amylin receptors is shown in Table 1. These results demonstrate that CGRP is a potent agonist at the only human $AMY_{1(a)}$ receptor. In rats, CGRP is also potent at $AMY_{3(a)}$ receptors (pEC_{50} rat amylin, rαCGRP: $rCT_{(a)}$ 8.11, 7.87; $rAMY_{1(a)}$ 9.74, 9.66; $rAMY_{3(a)}$ 9.97, 9.68) (Bailey *et al.*, 2012).

Antagonists are very useful tools for characterization of receptor pharmacology, provided a receptor-specific antagonist is available. The list of available antagonists targeting amylin receptors remains short and with only modest selectivity between CT and AMY receptors or between AMY receptors. These antagonists are all very similar, in that they are truncated forms of the full-length endogenous peptide or variants thereof (Figure 2). AC187, a salmon CT (sCT) and amylin chimeric peptide, has been widely used in the literature as an amylin receptor-specific antagonist (Figure 2). However, its discrimination of $hAMY_{1(a)}$ over $hCT_{(a)}$ was only 10-fold, with even less of a difference at rat receptors (Hay *et al.*, 2005; Bailey *et al.*, 2012). AC413, another sCT/amylin chimera, has a similar profile. Inconsistencies with rat $amylin_{8–37}$ have been reported, and it is considered a weak antagonist. $CGRP_{8–37}$ is a modestly effective antagonist of AMY receptors. The pharmacology of antagonists at AMY receptors is shown in Table 2. There are no known low MW antagonists of CT or AMY receptors. The highest affinity ligand is olcegepant, which was designed as a CGRP receptor antagonist, against the CLR/RAMP1 complex. This has a pA_2 of ~7.3 at $hAMY_{1(a)}$ (Hay *et al.*, 2006; Walker *et al.*, 2015).

Table 1

Peptide agonist pharmacology at calcitonin (CT) and amylin (AMY) receptors

Receptor	Agonist (pEC_{50}) hAMY	rAMY	hαCGRP	hβCGRP	hCT	sCT	Assay	Cell Line/ Tissue	References
$hCT_{(a)}$	–	7.06	7.20	–	8.88	9.78	cAMP	COS7	Udawela *et al.*, 2006a
	8.61	8.62	–	–	9.95	–	cAMP	COS7	Gingell *et al.*, 2014
	–	6.95	6.80	7.18	8.99	–	cAMP	COS7	Hay *et al.*, 2005
	8.27	–	–	–	9.00	10.2	cAMP	COS7	Albrandt *et al.*, 1995
	8.33	7.81	–	–	9.71	–	cAMP	HEK293S	Gingell *et al.*, 2014
	9.17	–	–	–	10.2	–	cAMP	RAEC	Muff *et al.*, 1999
	–	–	–	–	10.3	–	cAMP	COS7	Leuthauser *et al.*, 2000
	–	9.50	–	–	11.2	–	Dispersion	Melanophore	Armour *et al.*, 1999
	–	9.30	8.34	–	10.7	10.9	cAMP	COS7	Qi *et al.*, 2013
	–	9.39	8.11	–	12.0	–	cAMP	HEK293S	Qi *et al.*, 2013
	–	7.80	–	–	8.80	–	cAMP (30 min)	COS7	Morfis *et al.*, 2008
	–	8.26	–	–	9.61	–	cAMP (5 min)	COS7	Morfis *et al.*, 2008
	–	7.76	–	–	8.47	–	pERK1/2	COS7	Morfis *et al.*, 2008
	–	7.44	–	–	8.23	–	Ca^{2+}	COS7	Morfis *et al.*, 2008
	–	8.28	–	–	9.79	–	cAMP(30 min)	HEK293	Morfis *et al.*, 2008
	–	7.53	–	–	7.89	–	pERK1/2	HEK293	Morfis *et al.*, 2008
	–	7.51	–	–	8.07	–	Ca^{2+}	HEK293	Morfis *et al.*, 2008
	–	7.13	6.88	–	9.43	10.1	cAMP	COS7	Udawela *et al.*, 2006b
$rCT_{(a)}$	–	8.11	7.87 (rαCGRP)	7.54 (rβCGRP)	9.28 (rCT)	–	cAMP	COS7	Bailey *et al.*, 2012
$hCT_{(b)}$	–	7.12	7.09	–	8.75	10.22	cAMP	COS7	Udawela *et al.*, 2008
$hAMY_{(1a)}$	–	8.61	8.08	–	8.87	10.03	cAMP	COS7	Udawela *et al.*, 2006a
	9.71	9.90	–	–	9.96	–	cAMP	COS7	Gingell *et al.*, 2014
	–	9.12	8.70	9.16	8.93	–	cAMP	COS7	Hay *et al.*, 2005
	9.00	8.98	–	–	9.73	–	cAMP	HEK293S	Gingell *et al.*, 2014
	8.73	–	–	–	10.1	–	cAMP	RAEC	Muff *et al.*, 1999
	–	–	9.11	–	9.62	–	cAMP	COS7	Leuthauser *et al.*, 2000
	–	9.66	–	–	11.3	–	Dispersion	Melanophore	Armour *et al.*, 1999
	–	10.5	10.2	–	10.6	11.1	cAMP	COS7	Qi *et al.*, 2013
	–	10.1	9.16	–	–	–	cAMP	HEK293S	Qi *et al.*, 2013
	–	9.23	–	–	8.64	–	cAMP(30 min)	COS7	Morfis *et al.*, 2008
	–	9.76	–	–	9.28	–	cAMP (5 min)	COS7	Morfis *et al.*, 2008
	–	8.34	–	–	8.35	–	pERK1/2	COS7	Morfis *et al.*, 2008
	–	7.73	–	–	7.98	–	Ca^{2+}	COS7	Morfis *et al.*, 2008
	–	9.69	–	–	9.88	–	cAMP(30 min)	HEK293	Morfis *et al.*, 2008
	–	7.78	–	–	8.12	–	pERK1/2	HEK293	Morfis *et al.*, 2008
	–	8.22	–	–	7.65	–	Ca^{2+}	HEK293	Morfis *et al.*, 2008
	–	8.47	8.45	–	9.00	10.12	cAMP	COS7	Udawela *et al.*, 2006b
$rAMY_{1(a)}$	–	9.74	9.66 (rαCGRP)	8.87 (rβCBRP)	8.90 (rCT)	–	cAMP	COS7	Bailey *et al.*, 2012
$hAMY_{(1b)}$	–	7.92	8.10	–	9.93	9.77	cAMP	COS7	Udawela *et al.*, 2008
$hAMY_{(2a)}$	–	7.78	7.29	–	9.25	9.66	cAMP	COS7	Udawela *et al.*, 2006a
	9.11	8.86	–	–	9.93	–	cAMP	COS7	Gingell *et al.*, 2014
	8.27	8.47	–	–	9.64	–	cAMP	HEK293S	Gingell *et al.*, 2014
	8.73	–	–	–	10.3	–	cAMP	RAEC	Muff *et al.*, 1999
	–	9.90	–	–	11.4	–	Dispersion	Melanophore	Armour *et al.*, 1999
	–	8.25	–	–	8.82	–	cAMP(30 min)	COS7	Morfis *et al.*, 2008

(Continues)

Table 1 (Continued)

Receptor	Agonist (pEC_{50}) hAMY	rAMY	hαCGRP	hβCGRP	hCT	sCT	Assay	Cell Line/ Tissue	References
	–	8.53	–	–	9.27	–	cAMP (5 min)	COS7	Morfis *et al.*, 2008
	–	7.83	–	–	8.56	–	pERK1/2	COS7	Morfis *et al.*, 2008
	–	7.66	–	–	8.08	–	Ca^{2+}	COS7	Morfis *et al.*, 2008
	–	9.08	–	–	9.70	–	cAMP(30 min)	HEK293	Morfis *et al.*, 2008
	–	7.57	–	–	8.09	–	pERK1/2	HEK293	Morfis *et al.*, 2008
	–	7.44	–	–	8.11	–	Ca^{2+}	HEK293	Morfis *et al.*, 2008
	–	7.16	7.11	–	9.39	9.70	cAMP	COS7	Udawela *et al.*, 2006b
$hAMY_{2(b)}$	–	7.94	7.74	–	8.77	10.33	cAMP	COS7	Udawela *et al.*, 2008
$hAMY_{(3a)}$	9.60	9.47	–	–	9.54	–	cAMP	COS7	Gingell *et al.*, 2014
	–	8.63	7.60	7.67	8.02	–	cAMP	COS7	Hay *et al.*, 2005
	8.90	8.93	–	–	9.58	–	cAMP	HEK293S	Gingell *et al.*, 2014
	9.11	–	–	–	9.00	–	cAMP	RAEC	Muff *et al.*, 1999
	–	10.8	–	–	10.7	–	Dispersion	Melanophore	Armour *et al.*, 1999
	–	9.26	7.91	–	9.18	–	cAMP	COS7	Qi *et al.*, 2013
	–	10.2	9.17	–	12.1	–	cAMP	HEK293S	Qi *et al.*, 2013
	–	9.12	–	–	8.20	–	cAMP(30 min)	COS7	Morfis *et al.*, 2008
	–	9.57	–	–	9.03	–	cAMP (5 min)	COS7	Morfis *et al.*, 2008
	–	8.43	–	–	8.02	–	pERK1/2	COS7	Morfis *et al.*, 2008
	–	8.04	–	–	7.80	–	Ca^{2+}	COS7	Morfis *et al.*, 2008
	–	9.92	–	–	8.78	–	cAMP(30 min)	HEK293	Morfis *et al.*, 2008
	–	8.09	–	–	7.83	–	pERK1/2	HEK293	Morfis *et al.*, 2008
	–	8.23	–	–	7.73	–	Ca2+	HEK293	Morfis *et al.*, 2008
	–	8.61	7.62	–	8.17	9.58	cAMP	COS7	Udawela *et al.*, 2006b
$rAMY_{(3a)}$	–	9.97	9.68 (rαCGRP)	8.95 (rβCGRP)	8.30 (rCT)	–	cAMP	COS7	Bailey *et al.*, 2012
$hAMY_{3(b)}$	–	8.19	6.75	–	7.89	9.95	cAMP	COS7	Udawela *et al.*, 2008

Overview of amylin structure/function

A 'two-domain model of binding' has been proposed to describe family B GPCR peptide ligand receptor binding and activation. In this model, the peptide C-terminus binds to the moderate length extracellular N-terminus of the receptor, docking the peptide and optimally positioning the N-terminus of the peptide to bind to the upper transmembrane domain and extracellular loops of the receptor, ultimately causing receptor activation. In this model, the peptide C-terminus initiates binding to the receptor, and the N-terminal interactions activate the receptor (Hoare, 2005) (Figure 3). This model provides a useful framework for considering data on the structure–function relationships of amylin and its closely related family members. For example, the two-domain mode of binding for these peptides is supported by data with N-terminally truncated peptides, which bind the receptor but generally do not cause activation – the antagonists described above.

In fact, N-terminal deletions result in antagonists for all peptides in this family including human $amylin_{8–37}$, $\alpha CGRP_{8–37}$ and $AM_{22–52}$, supporting the notion of the importance of the N-terminus for receptor activation (Barwell *et al.*, 2012). Although binding affinity of all of these peptides is lower for each of these fragments compared with the parent peptide, this appears to be particularly the case for $amylin_{8–37}$. It has very low affinity for AMY receptors and is rarely used (Bailey *et al.*, 2012). The reasons for this are not known. In agreement with the two-domain model, a small 11mer fragment $h\alpha CGRP_{27–37}$ retains binding affinity in competition binding assays against receptor/RAMP ECD complexes (Moad and Pioszak, 2013).

N-terminal ring fragments of AM(15–22), αCGRP (1–8) and amylin (1–8) have been tested and are reportedly biologically active, inhibiting gastric acid secretion in rats, although with marked reductions in potency compared with their full-length equivalents (Rossowski *et al.*, 1997). Some activity of N-terminal fragments was preserved with cyclised fragments of human $amylin_{1–8}$, which retained the ability to stimulate rat fetal osteoblast proliferation and increase bone volume, albeit at very high concentrations (Kowalczyk *et al.*, 2012). In the native peptides, the C-terminus clearly needs to be present for full bioactivity. However, the N-terminal fragments with some activity could act as leads for further development. For example, there has been success with GLP-1 N-terminal fragments. This peptide, which mirrors many of

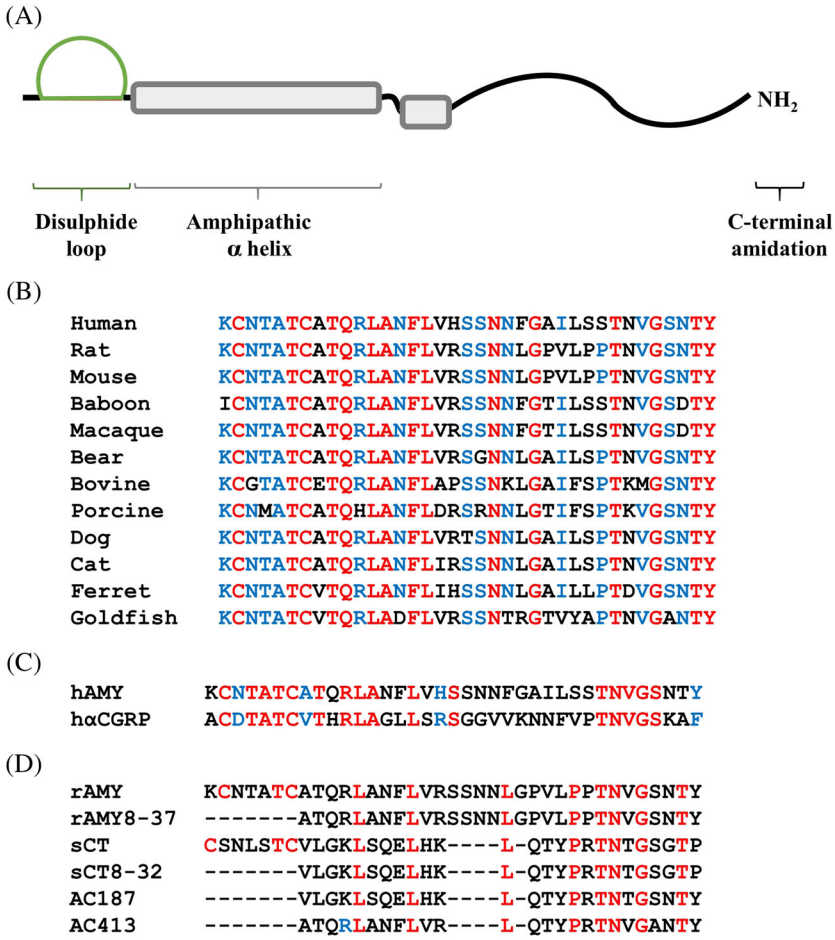

Figure 2

(A) Rat amylin general structure highlighting three important structural regions of the peptide: the N-terminal disulphide loop; the 7–17 amphipathic α helix followed by a turn, a small 20–24 residue α helix and 25–37 flexible loop; and an amidated C-terminus. (B)–(D) show amino acid sequence alignments of different species of amylin peptides, human amylin with CGRP and native rat amylin or salmon calcitonin with their truncated or chimera antagonist equivalents respectively. In these alignments, red residues are completely conserved residues across either the amylin species, between amylin/CGRP or between full-length peptides and antagonists. The blue residues are strongly similar and only differ between two amino acid residues, shown for (B) and (C) only.

the physiological effects of amylin on glucoregulation and satiety, is also a family B GPCR ligand. Modified N-terminal GLP-1 fragments have now been developed, which are very potent (Mapelli *et al.*, 2009; Hoang *et al.*, 2015).

We will outline the current understanding of the subregions of the amylin amino acid sequence and how this information may be used to refine drug development strategies. Given the overlap in activity of amylin and CGRP at the AMY_1 receptor, comparisons will be made between these two peptides.

Primary sequence

Within the CT family of peptides the N-terminus and C-terminus are the most highly conserved regions with more divergence in the mid-portions of each peptide. The same holds true for amylin across several species; strongly conserved termini with key variations in regions nearer the mid to C-terminal end of the peptide, suggesting an importance in retention of the N-terminal and C-terminal residues for biological activity (Figure 2B).

Certain amino acids across the CT family of peptides are of particular interest due to their strong conservation: particularly, Cys^2, Cys^7, Thr^6 and a C-terminal aromatic residue (except calcitonin, which has proline). The first residue is often small and uncharged, and the second is cysteine, which is always conserved in all species. The next four are highly conserved residues between amylin and CGRP sequences – Asp/Asn-Thr-Ala-Thr. Thr^6 appears to be conserved in all members of CT family across many species (Watkins *et al.*, 2013 (Figure 2C).

In amylin, the first two residues, lysine and cysteine, are strongly conserved from goldfish to humans (Figure 2B). The Ala-Thr-Cys sequence in positions 5, 6 and 7 are also highly conserved across species. Thr^9, Gln^{10}, a basic amino acid at position 11 (Arg or His in pig), Leu at position 12, Ala at position 13, Asn or Asp (goldfish) at 14, Phe at 15, Leu at 16, His/Arg/Pro at 18, Ser at 19 and Asn at position 21 are all mostly conserved across species. Positions 3 and 4 are mostly Asn and Thr, respectively,

Table 2

Peptide antagonist pharmacology at calcitonin (CT) and amylin (AMY) receptors

Receptor	Agonist	Antagonist (pK_b or pA_2) AC187	AC413	$rAMY_{8–37}$	$h\alpha CGRP_{8–37}$	$sCT_{8–32}$	Assay	Cell line	References
$hCT_{(a)}$	hAMY	7.25	–	–	–	8.09	cAMP	COS7	Gingell *et al.*, 2014
	hCT	7.15	6.94	<5	<5	8.17	cAMP	COS7	Hay *et al.*, 2005
	rAMY	6.89	7.48	–	–	8.22	cAMP	COS7	Hay *et al.*, 2005
	rAMY	8.85	–	–	–	8.95	cAMP	COS7	Qi *et al.*, 2013
	hCT	–	–	–	–	9.4	Dispersion	Melanophores	Armour *et al.*, 1999
	rAMY	–	–	–	–	9.25	Dispersion	Melanophores	Armour *et al.*, 1999
$rCT_{(a)}$	rAMY	7.78	8.09	<5	<5 (rat $_{8–37}$)	8.13	cAMP	COS7	Bailey *et al.*, 2012
$hAMY_{(1a)}$	hAMY	7.84	–	–	–	7.27	cAMP	COS7	Gingell *et al.*, 2014
	hCT	7.30	7.11	<5	<5	7.95	cAMP	COS7	Hay *et al.*, 2005
	rAMY	8.02	7.92	5.59	6.62	7.78	cAMP	COS7	Hay *et al.*, 2005
	rAMY	9.25	–	–	–	8.08	cAMP	COS7	Qi *et al.*, 2013
	hαCGRP	7.86	7.30	–	6.79	7.80	cAMP	COS7	Qi *et al.*, 2013
	hβCGRP	7.85	7.25	–	6.78	7.68	cAMP	COS7	Qi *et al.*, 2013
$hAMY_{(3a)}$	hAMY	8.30	–	–	–	8.21	cAMP	COS7	Gingell *et al.*, 2014
	hCT	7.37	6.83	<5	≤5	7.87	cAMP	COS7	Hay *et al.*, 2005
	rAMY	7.68	7.10	<5	6.17	7.92	cAMP	COS7	Hay *et al.*, 2005
	rAMY	–	–	<5	–	8.2	Dispersion	Melanophores	Armour *et al.*, 1999
	hCT	–	–	-	–	9.45	Dispersion	Melanophores	Armour *et al.*, 1999
$rAMY_{(1a)}$	rAMY	8.24	8.97	6.16	7.62, 7.07 (rat $_{8–37}$)	7.42	cAMP	COS7	Bailey *et al.*, 2012
$rAMY_{(3a)}$	rAMY	8.11	8.60	5.53	7.00 (rat $_{8–37}$)	8.17	cAMP	COS7	Bailey *et al.*, 2012

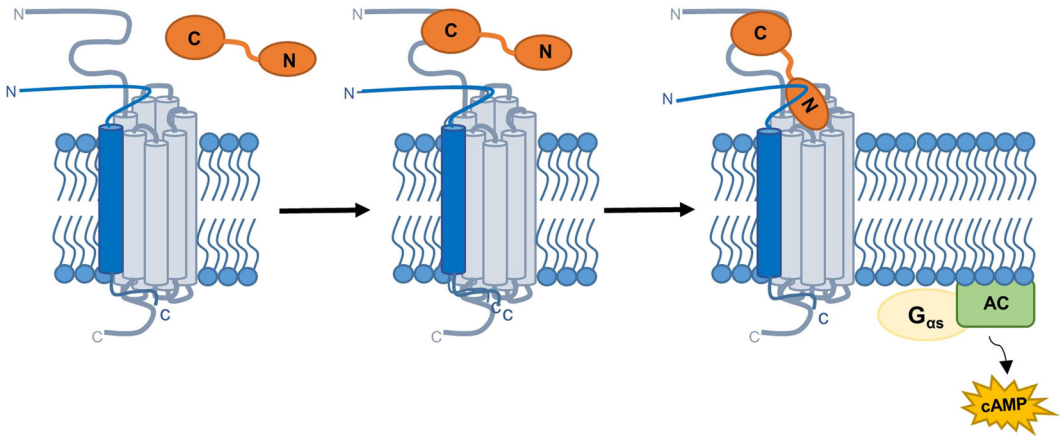

Figure 3

The two-domain model of peptide binding and receptor activation. From left to right, the peptide encounters the seven-transmembrane GPCR associated with a RAMP accessory protein in the cell membrane, the C-terminal end of the peptide binds to the extracellular N-terminus of the receptor complex and binding induces the alignment of the peptide N-terminus to the juxtamembrane region of the GPCR facilitating the activation of the G-protein, its subsequent association with adenylyl cyclase (AC) and downstream production of cAMP.

although this is not always the case as highlighted by cow and pig sequences. Position 8 is mainly Ala between species but is a Glu or Val in cow and goldfish respectively.

The mid-region of the peptide has the most divergence, and the residues are less conserved. Nearing the C-terminal portion of the peptide, the most highly conserved residues across species include Gly^{24}, Thr^{30}, Gly^{33}, Thr^{36} and the C-terminal aromatic Tyr^{37}. Small nonpolar residues occupy position 26 (Ile or Val), and prolines are common at position 29, but are replaced by a serine in humans and primates. Residue 32 is a small nonpolar

valine except in bovine species, replaced with a larger nonpolar methionine.

The most closely related peptide to amylin is CGRP (Figure 2C). It is particularly useful for comparing structure/function relationships with amylin given the ~50% sequence identity between them.

Disulphide loop/amidated C-terminus

Two biologically critical post-translational modifications in the CT family of peptides are also evident in amylin; the strongly conserved N-terminal disulphide loop between two cysteine residues and the amidated C-terminus (Figure 2A). These have distinctive roles in terms of receptor binding and activation.

The N-terminal disulphide loop, which has been termed the 'activation loop' is considered essential for receptor activation (Barwell *et al.*, 2012). Accordingly, linearized rat amylin with the C-terminal amide was 100-fold less potent at inhibiting glycogen synthesis in rat soleus muscle *in vitro* compared with the native peptide. The linear peptide also lacking the C-terminal amide lost all biological activity in this model (Roberts *et al.*, 1989). This finding was further reinforced using a rat fetal osteoblast proliferation assay, normally stimulated by wild-type rat amylin in the sub-nanomolar range. The peptide lacking both post-translational modifications was ineffective at eliciting osteoblast proliferation and analogues with only one of these modifications behaved as antagonists in this system (Cornish *et al.*, 1998). In CGRP, breaking the disulphide resulted in a linear peptide, which failed to increase blood flow or inhibit osteoclast resorption, whilst the intact full-length peptide retained these functions (Zaidi *et al.*, 1990). Destruction of the disulphide loop in hαCGRP also abolished bioactivity in rat atrial stimulation assays, further substantiating the importance of these features to the functionality of these peptides (Tippins *et al.*, 1986). Nevertheless, linear analogues of hαCGRP have been synthesized that challenge the paradigm of the unequivocal importance of the N-terminal disulphide 'activation loop'. In some assays, these analogues have been shown to retain binding affinity and/or activity either as full or partial agonists (Dennis *et al.*, 1989; Hay *et al.*, 2005; Bailey and Hay, 2006).

Synthesis of fragments of $hαCGRP_{27–37}$ and $AM_{37–52}$ lacking the C-terminal amide abolished binding of the peptide to receptor ECD complexes (Moad and Pioszak, 2013). In crystal structures of these peptides bound to their receptor ECDs, the importance of this C-terminal amide is clearly evident (Booe *et al.*, 2015). Similarly, when hCT is synthesized without its C-terminal amide or there is disruption of the N-terminal disulphide loop, drastic reductions in biological activity to induce hypocalcaemic effects are seen in rats (Rittel *et al.*, 1976).

These structural features in the amylin peptide have not recently been interrupted in the more classical physiological systems amylin is now known to act through, particularly their effect on producing glycaemic or gastric emptying effects or at defined receptors. However, these data suggest that both post-translational modifications have a strong influence on the affinity and efficacy of this family of peptides at their receptors. Alongside these elements, the primary sequence and resulting secondary structures of these peptides also play an important role in receptor recognition and activation.

Secondary structure

Amylin is considered an intrinsically disordered peptide lacking a structurally defined shape to perform its biological functions (He *et al.*, 2015). It has often been studied in the presence of sodium dodecyl sulfate (SDS) micelles and with other detergents in NMR spectroscopy for structural information on association with these membranes as a model of how it would act *in vivo* at a cellular and receptor interface (Watkins *et al.*, 2012 – see table therein). A caveat in interpreting these data is that SDS micelles and detergents potentially confound results given that they naturally induce α-helical secondary structures in favour of the native structure (Watkins *et al.*, 2012).

In the presence of dodecylphosphocholine (DPC) micelles using solution NMR, rat amylin was shown to have an N-terminal helix spanning residues 5–17, a short helix from 20 to 23 and a long flexible disordered loop from 24 to 37 (Nanga *et al.*, 2009); human amylin had a similar 1–17 residue helix (Nanga *et al.*, 2008). NMR and molecular dynamic simulation studies of the amyloidogenic and non-amyloidogenic sequences of human and rat amylin, respectively, suggested slightly different structural features with more α-helical content and fewer β sheets in rat amylin. The three main structural superfamilies observed in amyloidogenic amylin-included β-hairpin, helix-hairpin and helix-coil structures. Non-amyloidogenic sequences occupied only two of these superfamily structures including the helix-hairpin and helix-coil. The most dominant non-amyloidogenic structure was the helix-coil, whereby residues 1–7 formed a short turn-coil, an 8–17 α helix and a long turn coil from 18 to 37. The helix-hairpin fold had a 1–7 turn-coil followed by an 8–37 helix and a short β-hairpin. The structure only seen in amyloidogenic amylin was the β-hairpin motif comprising a β-strand from residues 9 to 17, a turn from 18 to 22 and another β-strand from 23 to 33 (Wu and Shea, 2013). Another molecular dynamic simulation study of both peptides in solution also indicated differences in secondary structure conformations with rat amylin adopting an N-terminal α helix and unstructured coil and human amylin with three conformations including an N-terminal α helix, β-hairpin and an unstructured coil (Chiu *et al.*, 2013).

NMR spectroscopy in zwitterionic DPC micelles of fragments of rat $amylin_{1–18}$ and human $amylin_{1–18}$ show that they have similar secondary structures with a 1–17 helix with a turn from Cys^7 to the N-terminus. These fragments only differ at position 18 (His, Arg in rat). Regardless, these fragments adopt different orientations with rat amylin staying at the membrane surface whilst human amylin buries deeper within the membrane. In acidic environments, protonating the His^{18} of human $AMY_{1–19}$ changes its membrane orientation to mimic that of rat amylin, suggesting the deprotonated state of His^{18} in neutral pH conditions may play a role in cell membrane or receptor interactions (Nanga *et al.*, 2008).

The helix-turn-disorder structures seem to be a theme throughout the CT family of peptides. Circular dichroism (CD) spectroscopy of human amylin and hαCGRP in aqueous

solution reveal largely unstructured conformations. When SDS micelles were added, helical content and overall secondary structure of both peptides increased (Saldanha and Mahadevan, 1991). Introducing solvents such as TFE also cause α-helical secondary structure increases from 20% to 70% for hαCGRP and hβCGRP. Fragment hαCGRP$_{8–37}$ had less helix content under both aqueous and TFE conditions compared with full-length hαCGRP suggesting that the first seven N-terminal residues may stabilize the helix (Hubbard *et al.*, 1991).

Amylin appears to be intrinsically disordered in aqueous solution, which suggests that it is likely to sample several different secondary conformations in physiological fluids with perhaps a more defined structure when interacting with its receptors. We must be mindful of the limitations of CD and NMR structural information considering solvent and artificial membrane influences on secondary structure. Also, the majority of the data available are from related peptides in the family as opposed to amylin itself, given its insolubility and fibrillogenic properties. Until crystal structures of the amylin peptide bound to one of its receptors become available, only inferences can be drawn on the data available, regarding its native secondary structure.

Structure/function – detailed information

Loop residues 1–7

An alanine scan of the non-cysteine residues in the N-terminal loop of rat amylin was undertaken whereby Lys^{1}, Asn^{3}, Thr^{4} and Thr^{6} within the loop were individually replaced, creating four alanine analogues. Each was tested for binding in rat nucleus accumbens membranes and for *in vivo* activity in fasted mice to reduce food intake. Alanine analogues 1, 3 and 4 retained binding and their *in vivo* anorexigenic activity. However, whilst Thr^{6} retained its binding ability, this analogue was no longer able to reduce food intake (Roth *et al.*, 2008). Initial modification of rat amylin Lys^{1} by iodination was not well tolerated, resulting in large reductions in binding affinity in rat liver plasma membranes, possibly due to steric interruption of interactions between the peptide N-terminus and its receptor. However, modification of Lys^{1} with the addition of a biotin moiety was accommodated when an eight-atom spanning bridge was included between the Lys-NH_2 group and the biotin C-terminus. The resulting analogue retained similar activity to rat amylin (Chantry *et al.*, 1992). The activity of any of these analogues at defined amylin receptor subtypes is not known.

Nevertheless, the limited data have parallels with CGRP. Residue mutations at positions 1, 3 and 4 to alanine were also unremarkable, with modest effects on binding or receptor activation. However, substitution of residues Ala^{5} or Thr^{6} within the disulphide loop with other amino acids was detrimental. This is perhaps unsurprising given the strong conservation of these residues across species in CGRP and the absolute conservation of Thr^{6} across the CGRP family of peptides (Hay *et al.*, 2014). Further details on the structure–function of this region of CGRP have recently been reviewed and should be consulted for more detailed information on modifications to the CGRP N-terminus (Watkins *et al.*, 2013).

These findings seem to challenge a strict two-domain model paradigm. Only one of the proposed 'activation loop' residues in rat amylin affected *in vivo* activity (Thr^{6}), and only two resulted in potency reductions for hαCGRP (Ala^{5}, Thr^{6}). These findings suggest that residues beyond this loop are also important for function, perhaps within the helical region.

Helix residues 8–18

All CGRP family peptides are proposed to possess an amphipathic helix comprising approximately 10 amino acids in length. Molecular dynamic simulations of sCT predict a helix spanning residues 9–19 (Amodeo *et al.*, 1999). Helical analogues of a rat amylin/sCT chimera were synthesized to ascertain the role of the amphipathic helix in peptide activity. Shortening of the helix resulted in a loss of efficacy to reduce food intake in mice *in vivo*. Conversely, lengthening the helix tended to retain or even enhance the potency of these analogues and improving the hydrophobic face of the helix increased peptide efficacy *in vivo* (Roth *et al.*, 2008). It is possible that these effects are due to increasing α-helical contacts with the peptide at the receptor interface or further stabilization of the peptide secondary structure.

Deletion of Leu^{16} from sCT resulted in large reductions in potency in hypocalcaemic assays and adenylate cyclase stimulation in T47D human breast cancer cells. Des-Leu^{16}-sCT partially retained binding, but the corresponding deletion analogue (Phe^{16}) of hCT lost nearly all the binding. In most CT species, position 16 is a Leu with the exception of hCT, where it is a Phe. In the CGRP family, amylin and CGRP have a strongly conserved Leu^{16}, across species. Deletion of this residue in CT perhaps has structural consequences on the stability of the amphipathic helix and the ability of the peptide to effectively interact with the receptor (Findlay *et al.*, 1983).

CGRP is proposed to possess an amphipathic helix spanning residues 8–18 (Lynch and Kaiser, 1988). Single-residue alanine analogues at positions 8 and 9 for Val and Thr, respectively, only resulted in small reductions in potency (Hay *et al.*, 2014). Whilst conserved across CGRP species, these residues are much more divergent in the CGRP family as a whole. Mutation of Arg^{11} or Leu^{12} to alanine in hαCGRP$_{8–37}$ had more substantial effects on affinity, perhaps due to reduced α-helical content. In amylin sequences, Arg^{11} is highly conserved with only the pig possessing a histidine at this position. Position 12 is quite conserved as Leu across the CGRP family, indicating it is likely to play an important role in structure/function relationships within the family (Howitt *et al.*, 2003).

It is evident that residues important for binding are present in N-terminal regions of these peptides and are not confined to the C-terminus. Mutations to the mid-peptide amphipathic helical regions may interrupt this important secondary structure and impact affinity and/or efficacy (Mimeault *et al.*, 1992).

Small helix residues 19–24: the 'amyloidogenic core'

Many analogues of amylin have been studied in order to investigate their role in amyloid formation *in vitro* (e.g.

Westermark *et al.*, 1990; Akter *et al.*, 2016). Unfortunately, they have not been interrogated for their contribution to the biological activity of amylin and thus will not be discussed in this review. Structure/function relationships will be analysed according to experiments performed on related peptides where data are available.

Alanine analogues of $h\alpha CGRP_{8-37}$ replacing Ser^{17}, Gly^{20} and Gly^{21} were well tolerated (Boulanger *et al.*, 1996). When Arg^{11} or Arg^{18} of $h\alpha CGRP_{8-37}$ were replaced with serines, only minimal reductions in binding affinity were observed. In the double mutant, affinity was reduced by 1000-fold. Replacing these residues with positively charged counterparts (Glu^{11} or Glu^{18} of $h\alpha CGRP_{8-37}$) also significantly affected binding (Howitt *et al.*, 2003). Therefore, at least one of the negatively charged Arg residues in this region of the peptide is needed for presumably important binding contacts with the receptor. In amylin species, the residue at position 18 is less conserved than in CGRP and is an Arg with the exception of the human variant (His^{18}) and the cow (Pro^{18}).

Flexible loop residues 25–37

Structure/function relationships of the C-terminal most residues of amylin have not been extensively investigated. However, a recent study investigated C-terminal fragments of sCT and a rat amylin/sCT chimera (AC413) in binding to the ECDs of CTR and amylin receptors (Lee *et al.*, 2016). Alanine replacements highlighted residues important for binding. In the AC413 fragment, critical residues for binding were the amylin residues Thr^{30}, Val^{32} and Gly^{33}. The aromatic Tyr^{37} did not seem to be important.

The crystal structures for C-terminal fragment peptides, a $CGRP_{27-37}$ analogue and AM_{25-52} bound to their equivalent RAMP and CLR ECDs, combined with alanine scanning and residue swapping between the peptides, have revealed additional helpful structure/function information for peptide-receptor binding. Important residues for $CGRP_{27-37}$ binding included Thr^{30}, Val^{32} and Phe^{37} in addition to an intact C-terminal amide. The recent AC413 data from Lee *et al.*, illustrate the conservation of mechanism for Thr^{30} and Val^{32}, which are conserved between CGRP and amylin (Figure 2C). Positions 30 and 32 are also identical threonine and valine residues across amylin species (apart from cow, which is a methionine at position 32). The final C-terminal residue in all amylin species is an aromatic tyrosine, similar to the bulky hydrophobic character of CGRP's phenylalanine. This suggests that both may make important hydrophobic interactions with receptor ECDs but alanine substitution did not greatly affect amylin affinity, unlike the equivalent mutation for CGRP (Moad and Pioszak, 2013; Booe *et al.*, 2015; Lee *et al.*, 2016). Other studies have also shown the importance of the C-terminal ring in CGRP (Dumont *et al.*, 1997; Smith *et al.*, 2003).

For AM_{25-52}, residues Pro^{43}, Lys^{46}, Ile^{47}, Gly^{51}, Tyr^{52} and the C-terminal amidation were critical for activity, further reinforcing the importance of the C-terminal aromatic residue. In sCT, the C-terminal proline was also critical (Lee *et al.*, 2016). It is possible that there are subtly different mechanisms at play in receptor binding between CGRP/AM/CT and amylin.

With regard to secondary structure, both AM and CGRP C-terminal fragments were largely disordered in the crystal structures with only AM_{25-52} displaying a small α-helical turn (Booe *et al.*, 2015). These data agree with NMR and CD structural determinations at the C-terminus for hαCGRP and hAMY, as previously described. These structural features are those observed from truncated peptides. The remaining residues of CGRP and AM could potentially affect the secondary structure of these peptides. Important residue interactions that may impart and influence secondary structure and peptide folding cannot be ruled out from the existing data.

Translating structure–function information into novel peptides

Structural manipulations and investigations have provided some clues into important residues and regions for amylin peptide/receptor interactions. However, to further guide the design of novel and improved amylin mimetics, information concerning the physiological half-life of amylin and break-down mechanisms must be considered for further exploitation.

Amylin metabolism

Amylin metabolism appears to be via a combination of renal clearance and proteolysis to generate a variety of metabolites, such as a des-Lys metabolite, which is active, and other cleavage products that are unlikely to generate active peptide fragments (Nakazato *et al.*, 1990; Watschinger *et al.*, 1992; Leckstrom *et al.*, 1997; Vine *et al.*, 1998). Amylin has a circulating plasma half-life of approximately 13 min in rats following an intravenous bolus injection (Young, 2005).

Radioimmunoassays of homogenized human pancreatic tissue showed it to contain human $amylin_{1-37}$ as the major molecular form with two additional C-terminal fragments: $amylin_{17-37}$ and $amylin_{24-37}$ (Miyazato *et al.*, 1994). This suggests that human amylin processing may occur between residues Leu^{16} and Val^{17} and between residues Phe^{23} and Gly^{24} forming these major by-products. In rats, mice and cats, $amylin_{1-37}$ and $amylin_{19-37}$ were detected with the latter as the major form identified. Therefore, processing occurs between Arg^{18} and Ser^{19}, unlike the processing in humans (Miyazato *et al.*, 1992). In rat plasma and pancreas, N-terminal fragments were also detected along with full-length amylin; $amylin_{1-16}$, $amylin_{1-17}$. Interestingly, these N-terminal fragments were not present in human pancreatic tissue (Miyazato *et al.*, 1994). The data available for amylin clearance are limited; however, some data suggest it can be processed by insulin-degrading enzyme (IDE) between amino acids Phe^{15}/Leu^{16} and His^{18}/Leu^{19} (Guo *et al.*, 2010); the latter of which is consistent with the presence of amylin fragments 19–37 in cats, rats and mice. Other cleavage sites of human amylin by IDE were revealed between residues Arg^{11}/Leu^{12} and Asn^{31}/Val^{32} (Shen *et al.*, 2006). Also, injecting obese and lean mice with selective IDE inhibitors resulted in increased plasma amylin levels and slowed gastric emptying compared with control animals (Bellia and Grasso, 2014; Maianti *et al.*, 2014). CGRP is cleaved at position 16 yielding the 17–37 fragment, which have been shown to weakly antagonize the actions of the parent peptide (Miyazato *et al.*, 1994).

In humans, amylin circulates in both glycosylated and non-glycosylated forms, with the latter considered the biologically active species (Fineman *et al.*, 1997). Naturally occurring O-linked glycosylation is found on N-terminal threonine residues, Thr^6 and/or Thr^9, completely abolishing agonist activity in rat soleus muscle. The modification of Thr^6 in particular given its conservation and location within the N-terminal 'activation loop' may explain the loss of activity.

Given the importance of understanding amylin metabolism, further studies using modern analytical techniques, such as sensitive mass spectrometry, would provide further insight into peptide residues to focus on in attempts to prolong plasma half-life or the half-life of amylin analogues. Improvements to the half-life of amylin and amylin mimetics could be clinically beneficial, and a range of strategies are being employed to generate modified peptides either with a longer duration of action, greater solubility or increased efficacy as outlined below.

Pramlintide

The advantageous properties of the prolines in the rat amylin sequence resulted in the creation of the amylin mimetic pramlintide. Pramlintide is a chimeric peptide composed of the human amylin primary sequence with three proline substitutions from the rat amylin primary sequence: $Ala^{25}Pro$, $Ser^{28}Pro$ and $Ser^{29}Pro$ (Figures 2, 4). Pramlintide retains all of the beneficial actions of native amylin without the disadvantages of amyloid formation and cytotoxicity (Schmitz *et al.*, 1997). Pramlintide is an FDA-approved adjunctive treatment to insulin for type 1 and type 2 diabetes, with further scope as an anti-obesity therapy. Unfortunately, pramlintide must be buffered at an acidic pH of 4 in order to retain solubility. Because insulin is buffered at a neutral pH, the two cannot be mixed in a single formulation (Nonoyama *et al.*, 2008). Therefore, patients receiving both pramlintide and insulin require two separate injections before meals, which limits compliance. Considerable efforts have been employed to improve both the pharmacokinetics and/or formulation limitations of pramlintide.

Glycosylated pramlintide

A common technique to improve drug half-lives is by introducing oligosaccharides onto peptide residues. Asparagine-linked glycosylation is possible at the six Asn residues present on the pramlintide peptide: Asn^3, Asn^{14}, Asn^{21}, Asn^{22}, Asn^{31} and Asn^{35}. Three sugars of increasing size (GlcNac, penta and undecasaccharides) were enzymically added at each Asn and screened for retention of biological activity in COS7 cells transfected with $hAMY_{1(a)}$ or $hAMY_{3(a)}$ receptors for cAMP production. An analogue is illustrated in Figure 4. The analogues with glycosylation closer to the C-terminus were better tolerated with only moderate reductions in potency at both receptors (Kowalczyk *et al.*, 2014). Enzymatically added GlcNAc residues on Asn^3 and Asn^{21} are also biologically active in CHO-K1 cells at calcium signalling; however, even a GlcNAc on Asn^3 reduced efficacy and penta/undecasaccharides had 10-fold and 20-fold losses in activity respectively. *In vivo*, rats treated with glycosylated Asn^{21} pramlintide with GlcNAc, and the pentasaccharide, but not the undecasaccharide, were effective in an oral glucose tolerance test. This illustrates that glycosylated pramlintide is active but supports the notion that larger sugars may disrupt secondary structure and/or sterically interfere with peptide activity (Tomabechi *et al.*, 2013), at least with the positions utilized and synthetic approach used thus far.

Chimeras

Davalintide is a potent amylin mimetic agonist retaining the favourable biological effects of amylin. It is a chimera of the primary sequences of rat amylin and sCT (Figures 2, 4). Davalintide is 32 amino acids in length and also contains the conserved disulphide bridge between Cys^2 and Cys^7 with an amidated C-terminus. Competition binding assays in rat nucleus accumbens membranes and production of cAMP in rat insulinoma cells revealed that davalintide remained equipotent with rat amylin (Mack *et al.*, 2010).

Acute and prolonged administration of davalintide in rodents produced dose-dependent reductions of food intake and fatpad-specific weight loss with reductions in gastric emptying rates and glucagon production (Mack *et al.*, 2011). Like amylin, these effects were not due to increased locomotor activity, suggesting activation of similar centrally mediated metabolic pathways. Lesioning of the area postrema further confirmed this, as the food-suppressive and weight-loss effects of both davalintide and rat amylin were abolished. Expression of c-fos overlapped in the same brain regions for rat amylin and davalintide; however, expression was prolonged for davalintide, which lasted 8 h compared with 2 h with rat amylin (Mack *et al.*, 2010).

With a half-life of 26 min for davalintide compared with 17 min for rat amylin, a reduced rate of renal clearance was considered unlikely to be the reason for this longer half-life. This disparity was attributed to reduced dissociation rates of the davalintide observed in membrane preparations, as their association rates were similar (Mack *et al.*, 2011). This effect is likely to be attributable to the sCT sequence within the peptide, where the degree of helical secondary structure in sCT appears to influence peptide binding kinetics (Hilton *et al.*, 2000).

Phybrids

The concept of combination therapies has been brought a step further with peptide hybrids or 'phybrids', combining two distinct peptides with beneficial metabolic effects, which may additively influence weight loss and/or glucoregulation by targeting two distinct receptors with complementary biological activity. Two such compounds, AC164204 and AC164209, produced by Trevaskis *et al.* (2013) have achieved this. The GLP-1 receptor agonist exenatide analogue AC3082 C-terminus was linked covalently to the N-terminus of amylin chimera $des\text{-}Lys^1$-davalintide (Figure 4). AC164204 and AC164209 differ by the type of linker used between peptides with a β-Ala-β-Ala and Gly-Gly-Gly spacer respectively. Davalintide was C-terminally amidated, and after ligation was oxidized to produce the N-terminal 2–7 disulphide loop (Trevaskis *et al.*, 2013). *In vitro*, HEK293 cells transfected with $hCT_{(a)}$ or rat thyroid carcinoma cells expressing the GLP-1 receptor were used to measure cAMP production. Both phybrids acted as agonists at these receptors with potencies similar to their parent peptides, albeit with blunted potency at the $hCT_{(a)}$ probably due to the N-terminal linker attachment to davalintide and/or other potential steric hindrance

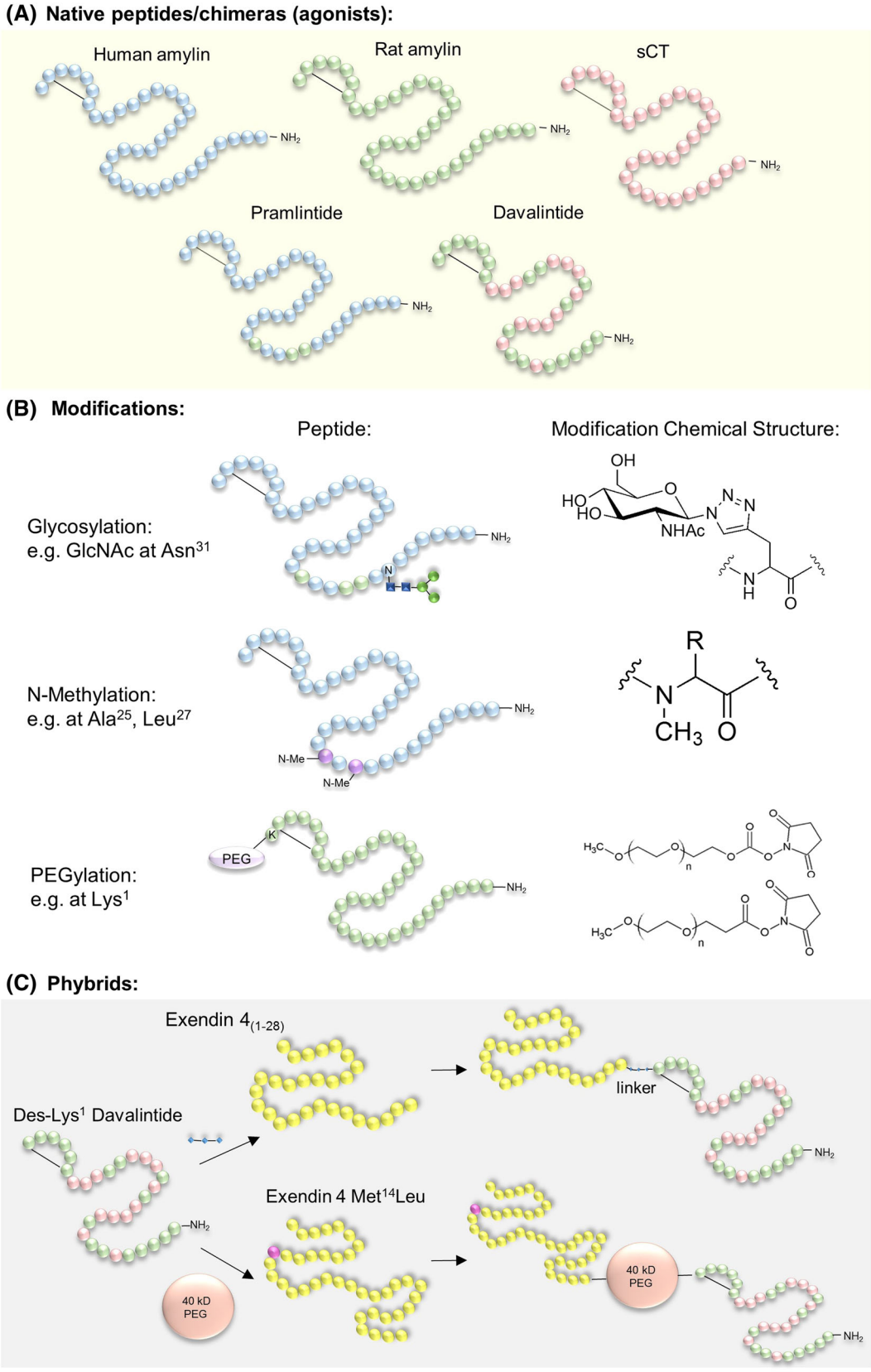

Figure 4

Amylin and amylin mimetic peptides, including examples of chimeras (A), peptide modifications (B) and phybrids (C).

imposed by this modification. The hAMY receptor(s) themselves were not explored here and require further analysis. Both acute i.p. administration and sustained infusions of both analogues dose-dependently improved HbA1c levels after an oral glucose tolerance test in normal, obese and diabetic mice. Compared with the parent peptides administered alone, sustained infusions resulted in greater appetite suppression and weight loss in *ob/ob* mice and rats with diet-induced obesity (DIO). Fat pad analysis of DIO rats revealed smaller fat pads with a maintenance of lean mass (Trevaskis *et al.*, 2013).

N-methylation

The addition of methyl groups to amines in peptides (N-methylation) is also a technique employed to improve peptide pharmacokinetics and/or formulation. N-methylated derivatives of human amylin with methylation at positions Gly^{24} and Ile^{26} had improved solubility. They also retained receptor activation, although with reductions in binding and potency when tested in MCF-7 cells expressing amylin-responsive receptors. While MCF-7 cells are commonly used models for amylin receptor pharmacology, the exact composition of their receptors and RAMP expression is currently undefined. To improve potency and binding parameters, derivatives were created with methylation at position Ala^{25} and Leu^{27}, Phe^{23} and Ala^{25} or Ile^{26} and Leu^{27}, which all had full-agonist activity for cAMP production in MCF-7 cells. The peptide with methylation at Ala^{25} and Leu^{27} was three-fold more potent than human amylin, while the other analogues were equipotent. All derivatives also displayed improved half-lives over human amylin and pramlintide (Yan *et al.*, 2013).

PEGylation

PEGylation is the covalent attachment of polyethylene glycol (PEG) polymers to peptides or proteins and is used to improve the pharmacokinetic profiles of peptides by potentially increasing the half-life by inhibiting renal clearance and/or protease degradation, reducing immunogenicity and/or increasing solubility (Pisal *et al.*, 2010).

Methoxyl monoPEGylated and diPEGylated murine amylin were synthesized with the PEGylated residue being the N-terminal lysine at position 1 due to the availability of its two free amino groups (Figure 4). Subcutaneous administration of murine amylin and both of its PEGylated derivatives in mice caused equivalent reductions in blood glucose levels. However, both derivatives displayed a prolonged duration of action, reaching maximal effect at 5 h post-administration compared with 2 h for unmodified amylin. The hypoglycaemic actions of unmodified amylin lasted a total of 5 h whereas both PEGylated amylin derivatives lasted a full 24 h (Guterres *et al.*, 2013). The retained bioactivity of both PEGylated conjugates reveals that these modifications did not hinder the ability of the peptide to activate its receptor(s), despite the additions of large PEG groups at Lys^{1}. The longer duration of action of these conjugates also validate this technique in improving the half-life of amylin (Guterres *et al.*, 2013). These conjugates were also effective in reducing glycaemia when subcutaneously administered in rats with consistently prolonged durations of action over that of unmodified amylin (Guerreiro *et al.*, 2013).

PEGylated hybrids

To further improve the half-life and stability of the aforementioned hybrids, the C-terminus of exenatide analogue AC3174 was linked to the N-terminus of the davalintide with a 40 kD PEG between the two peptides (Figure 4). Activity was analysed *in vitro* at the CTR by measuring cAMP accumulation in transfected HEK293 cells and at rat thyroid C-cells, which endogenously express GLP-1 receptors. It activated both receptors in the low-nM concentration range (Sun *et al.*, 2013). *In vivo*, dose-dependent reductions in blood-glucose levels and weight loss were seen following subcutaneous injections in female rats with longer-lasting effects compared with rat amylin, sustained for up to 4–5 days. In DIO rats, the conjugated analogue dose-dependently reduced food intake and body weight with a half-life of 27 h. The estimated human half-life calculated from this was determined to be approximately 100 h and amenable to a once-weekly dosing regimen (Sun *et al.*, 2013).

Closing remarks

From available data thus far, it is becoming increasingly apparent that whilst the two-domain model of binding and activation for family B receptor peptide ligands is useful, this is only to a limited degree. N-terminal ring fragments retain biological activity (Rossowski *et al.*, 1997), and C-terminal fragments are often antagonists and retain binding to receptors (Barwell *et al.*, 2012) validating these facets of the model; however, the data are not always so cleanly defined. Also questionable is the degree of importance of the disulphide 'activation loop' and C-terminal tyrosine amide in the amylin peptide. The studies for amylin investigating their roles have not been carried out in biological assays with defined amylin receptors or in measuring canonical amylin-mediated physiological actions (Roberts *et al.*, 1989). With CGRP, breaking the N-terminal disulphide still resulted in partial agonists in some biological assays. There are suitable suggestions as to the secondary structure the native amylin peptide adopts although, in solution, it is likely to be disordered and capriciously change structure (He *et al.*, 2015). Information from CD and NMR studies utilize detergent membranes to mimic cellular membrane/peptide interactions, which are not ideal as they naturally instigate helical conformations, and these vary depending on solvent used and/or micellar composition (Watkins *et al.*, 2012). In order to be certain, crystal structures of amylin or pramlintide bound to an amylin receptor are needed. The crystal structures for a $CGRP_{27–37}$ analogue and $AM_{37–52}$ offer useful insights (Booe *et al.*, 2015) but fragments only tell part of the story, and N-terminal interactions with receptor juxtamembrane regions are excluded in these models.

The scope for peptide modification strategies to develop new amylin mimetics is substantial. However, to drive drug design and development, more information is needed to understand amylin and how it acts to elicit physiological responses and, thus, how its structure influences function. Metabolism and glucoregulation are enormously complex physiological processes requiring multifaceted hormonal

and enzymic responses. In the future, it is likely to be combination therapies that will be the most useful to effectively target diseases such as diabetes and obesity.

Conflict of interest

The authors declare no conflicts of interest.

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