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Amylin in vasodilation, energy expenditure and inflammation

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1. ABSTRACT

Metabolic syndrome significantly increases the incidence of atherosclerosis-related diseases including coronary artery disease, stroke, and type 2 diabetes. Recent progress has demonstrated that amylin, or islet amyloid polypeptide, is circulating multifunctional hormone and neuropeptide, which is co-secreted with insulin into the bloodstream by pancreatic beta cells and plays a very important role in regulating feeding, energy homeostasis and inflammation. Recent FDA approval of amylin analog pramlintide as a new drug for treating type 1 and 2 diabetes positions amylin in the spotlight. In this analytical review, I summarize the recent progress on amylin studies in the following sections: *1)* introduction to the molecular features of amylin; *2)* amylin's amyloidogenic and proinflammatory effects; *3)* a satiety hormone and new drug in increasing energy expenditure; and *4)* a vasodilator inducing hypotension and tachycardia; and *5)* a neuropeptide in depolarizing cholinergic neurons via closure of potassium channels. Continued improvement of our understanding on this multifunctional hormone would lead to future development of pramlintide as novel therapies for other inflammatory, hematological, metabolic, neurological and vascular diseases.

2. INTRODUCTION – MOLECULAR FEATURES

Metabolic syndrome (MS) is a group of risk factors, including hypertension, hyperglycemia, hyperlipidemia and central obesity, which significantly increase the incidence of atherosclerosis-related diseases including coronary artery disease, stroke, and type 2 diabetes(1). Atherosclerotic cardiovascular disease results in 80% of diabetic mortality and more than 75% of all hospitalizations for diabetic complications. The defining characteristic of metabolic syndrome includes insulin resistance, abdominal obesity, hypertension, and a common form of dyslipidaemia (raised triglycerides and decreased high-density lipoprotein cholesterol(HDL) with or without elevation of low-density lipoprotein cholesterol(LDL))(2). More than 27%(3) of the U.S. population suffers from metabolic syndrome, representing a national healthcare crisis. Current therapies are limited, which results from our relatively poor understanding of the molecular mechanisms underlying feeding regulation and energy expenditure. Therefore, significant improvement of our understanding on this aspect is required for the future development of novel therapeutics.

Recent progress reported that gastrointestinal neuropeptides play critical roles in regulating feeding,

Table 1. Receptor components and G-protein signaling of receptors in the calcitonin peptide family

Receptor	Components	G Protein
Calcitonin	*CALCR*	G_s/G_q
Amylin1	*CALCR + RAMP1*	G_s
Amylin2	*CALCR + RAMP2*	G_s
Amylin3	*CALCR + RAMP3*	G_s
Calcitonin gene related peptide	*CALCRL + RAMP1*	G_s/G_q
Adrenomedulin1	*CALCRL + RAMP2*	G_s
Adrenomedullin2	*CALCRL + RAMP3*	G_s

energy homeostasis and inflammation. Most gastrointestinal peptides including amylin, glucagon like peptide-1, peptide YY, and oxytomodulin are anorectic, and only ghrelin is an orexigenic peptide. Although there have been several excellent reviews in this topics (4, 5), there are needs to us to analyze the new discoveries (6, 7). In this review, we focus our efforts on analyzing the roles and mechanisms of amylin as anorectic gastrointestinal peptide hormone that regulates energy homeostasis and as well as a proinflammatory protein that regulates inflammation. In addition, we will also discuss the roles of amylin as a neuropeptide. The continued improvement of our understanding of how amylin functions could eventually lead to the development of novel therapeutics for metabolic syndrome-related myocardiac infarction and stroke.

Amylin, or islet amyloid polypeptide (IAPP), is a 37-amino acid peptide that is co-released with insulin into the bloodstream by pancreatic beta cells in response to nutrient stimuli. Amylin is part of the calcitonin gene family, which includes calcitonin, calcitonin gene-related peptide (CGRP), adrenomedullin, and intermedin/adrenomedullin 2. Calcitonin receptor stimulating peptide has recently been added to this family, but is not found in humans. Amylin gene (8) is on the short arm of human chromosome 12. Amylin is synthesized as a 89 amino acid preprohormone (proIAPP)(9), which is further cleaved to form amylin by prohormone convertases PC2 (10) and PC3(11) as shown in Figure 1. Further posttranslational processing includes amidation at the C terminal, and formation of a disulphide loop between cysteine-2 and cysteine-7 (12). In humans, amylin is 43% similar to CGRP, and 20% homologous with adrenomedullin (13). Calcitonin and CGRP are alternatively spliced products of the same calcitonin gene (14). Although human amylin is not homologous to calcitonin, sequence similarities exist between human amylin and salmon calcitonin in the N- and C-terminal regions (15, 16). This similarity with salmon calcitonin has been exploited in the development of amylin receptor antagonists, which are based on salmon calcitonin fragment 8-32.

The amylin receptor is composed of a calcitonin receptor heterodimerized with receptor activity modifying protein 1 (RAMP1) and RAMP3 (17). The members of RAMP subgroup are a subgroup of single transmembrane proteins that modifies the receptor affinity of the calcitonin receptor to bind amylin. RAMP proteins modifies the calcitonin receptor (CALCR) and calcitonin receptor like receptor (CALCRL) binding specificity and may modulate other functions such as receptor internalization and recycling and strength of activation of downstream signaling pathways (18). As shown in Table 1, receptors for the calcitonin peptide family are composed of combinations of CALCR or CALCRL with RAMP1, RAMP2, and RAMP3. In addition to modifying receptor binding affinity in the calcitonin peptide family, RAMPs have also been found to be involved in receptor trafficking. In a study of adrenomedullin receptors 2 (CALCRL+ RAMP3), the PDZ type I motif at the C terminus of RAMP3 has been found to interact with *N*-ethylmaleimide-sensitive factor and cause the CALCRL/RAMP3 complex to be targeted for recycling after internalization (19). RAMPs have been found to be required for the forward trafficking of the calcium sensing receptor to the plasma membrane (20).

3. AMYLOID DEPOSIT OF AMYLIN IS PATHOGENIC AND PROINFLAMMATORY IN MULTIPLE ORGANS

One of the important pathophysiological roles of amylin is related to its feature of aggregation. Protein aggregation, with the generation of small amyloid deposits in specific organs, also occurs outside the central nervous system and often is associated with increased cell death. For example, it has been accepted that local cerebral aggregation of the small peptide Abeta is involved in the pathogenesis of Alzheimer's disease. Comparing to peptide Abeta, amylin (IAPP) is a lesser known but common localized amyloid fibril-forming protein. Amylin aggregates and induces the depletion of islet beta-cells via cell death and beta-cell dysfunction as well as inciting islet inflammation in type 2 diabetes and in islets transplanted into type 1 diabetic subjects. Initial amyloid deposition occurs intracellularly and parts of this amyloid consist of proIAPP (21). Amyloid formation into toxic polymers by amylin in pancreatic β-cells is a characteristic of the progression of clinical diabetes (22). A number of other stressors, including insulin resistance and hyperglycaemia, may contribute to amyloid formation by increasing amylin production by the beta-cell(23). Transgenic mouse islet studies showed that amyloid was detected after both one and three weeks of culture in the transgenic mouse islets and the encapsulated islets were most affected(24).

Using molecular epidemiology approach, a report showed that a short-term diet and exercise intervention ameliorates inflammation and markers of metabolic health in overweight/obese children. The results showed that interleukin (IL)-6, IL-8, amylin, tumor necrosis factor (TNF)alpha, plasminogen activator inhibitor (PAI-1), resistin, leptin, insulin, and IL-1 receptor antagonist (IL-1ra) decreased and adiponectin increased, suggesting that amylin was decreased along other proinflammatory cytokines(25). Moreover, a recent population-based study measured plasma amylin, inflammatory markers (C-reactive protein [CRP] and IL-6), insulin, glucose and lipid profiles in 1,011 Chinese men and women aged 35-54 years. The results showed that amylin is strongly associated with inflammatory markers and metabolic syndrome. The amylin-metabolic syndrome association is independent of established risk factors of

Figure 1. Processing of proamylin into amylin. The amino acid sequence of human pro-amylin with the cleavage site for PC2 at the NH2 terminus and the cleavage site for PC1/3 at the COOH terminus, indicated by arrows. The KR residues (blue) that remain at the COOH terminus after PC1/3 processing are removed by carboxypeptidase E. This event exposes the glycine residue that is used for COOH-terminal amidation. Above is a cartoon of amylin in grey with the intramolecular S-S bond between residues 2–7 and the amidated COOH terminus.

metabolic syndrome, including obesity, inflammatory markers and insulin resistance. However, the question regarding the causal role of hyperamylinemia in the development of metabolic syndrome remains to be answered(26). In addition to the critical role of amylin played in islet amyloidosis and in the development of beta-cell dysfunction in patients with diabetes, a recent report showed that a renal amylin deposition was found in patients with diabetic nephropathy and that the deposition was associated with disease severity(27).

The toxicity of the amyloid formation has been recently discovered to be due to mitochondrial dysfunction(28). The inhibitor for mitochondrial enzyme Abeta binding alcohol dehydrogenase AG18051 confers partial protection from amylin toxicity(29). The study of calcium signals in amylin overexpressing cells demonstrated an absence of response to glucose and also to the potassium channel blocker tolbutamide, indicating a defect in ATP-sensitive potassium (K(ATP)) channels. Amylin overexpression inhibits insulin and IAPP secretion in response to glucose affecting the activity of K(ATP) channels, potentially via disrupt membranes of the secretory pathway and mitochondrial membranes(30). Interestingly, amylin-overexpressing cells showed a greater maximal respiratory capacity than control cells. These data were confirmed by an increased mitochondrial membrane potential in amylin-overexpressing cells under glucose stimulation, leading to an elevated reactive oxygen species level as compared with control cells. Increased mitochondrial metabolism may be a compensatory response to counteract the secretory defect of beta-cells (31).

A recent report showed that IAPP-induced islet chemokine secretion promotes macrophage recruitment and that IL-1 receptor (IL-1R)/myeloid differentiation primary response gene 88 (MyD88), but not Toll-like receptor 2 (TLR2) or TLR4 signaling is required for maximal macrophage responsiveness to prefibrillar IAPP. These data raise the possibility that islet amyloid-induced inflammation contributes to β cell dysfunction in type 2 diabetes and islet transplantation(32). For the detailed molecular mechanisms underlying amylin's role in triggering inflammation, recent reports suggest detailed pathways(26, 33). Atherosclerosis is the cause of morbiditiy for 70% of patients with type 2 diabetes. In both of these diseases, a newly characterized inflammatory caspase-1-activating protein complex termed as inflammasome is stimulated via inappropriate oligomerization or misfolding proteins(34) including amylin (IAPP)(35) to activate interleukin-1β (IL-1β) and IL-18, which are pathogenic inflammatory cytokines(36). Triggers for the inflammasome assembly are obesity-related factors, such as cholesterol crystals in atherosclerosis, or hyperglycemia, ceramides, and amylin in type 2 diabetes. Of note, therapeutics that target IL-1β in clinical trials for type 2 diabetes might also decrease the incidence of atherosclerosis(37). Moreover, for the molecular mechanisms underlying amylin's upregulation in response to inflammatory stimuli, a recent report suggest that proinflammatory cytokine TNF-alpha acutely induces amylin gene expression in beta-cells through multiple signaling pathways, possibly contributing to amylin elevation in acute inflammation-related pancreatic disorders(38). These pathways includes the protein kinase C (PKC)zeta – extracellular signal-regulated kinase (ERK)1/2 - activator protein (AP) pathway and PKC(zeta)-c-Jun-N-terminal kinase (JNK)-AP1 pathways, phosphatidylinositol 3 kinase-NF-kappaB signalling pathway and enhanced human amylin promoter activation through NF-kappaB and AP1. Despite these promising discoveries, future studies are needed to determine whether amylin could act as a proinflammatory cytokine, similar to other proinflammatory cytokines TNF-alpha, interferon-gamma and IL-1beta, to directly initiate its receptor-specific inflammatory signaling.

4. AMYLIN IS A SATIETY HORMONE AND NEW DRUG THAT INCREASES ENERGY EXPENDITURE

In the blood stream, as a satiety hormone, amylin reduces appetite (39). Amylin limits a meal size by promoting meal-ending satiation, which depends on a direct

action in the amylin receptor-rich area postrema in the brainstem. In addition, amylin acts as an adiposity signal. Plasma amylin concentrations are found to be higher in overweight/obese participants than normal-weight controls. Chronic infusion of amylin into the brain reduces body weight gain and adiposity. Chronic infusion of an amylin receptor antagonist into the brain increases body adiposity. Moreover, amylin slows gastric emptying (15), and decreases postprandial glucagon secretion (40). Furthermore, amylin increases energy expenditure, which is associated with increased fat oxidation supplying energy(5). This effect occurs under various experimental conditions after peripheral and central administration. Its association with type II diabetes in humans has been well characterized (12, 41-43). This propensity appears to be species specific, and is seen in a relatively restricted number of mammalian species. Amylin-containing nerve fibers are found in the spinal trigeminal tract, NTS, and the area postrema. Amylin effects in the area postrema are related to its anoretic effects. Specifically, post-prandial amylin release leads to activation of the subfornical organ and the area postrema in the central nervous system (44), resulting in decreased food intake. Studies on the central effects of amylin have found that amylin may play a role as an adiposity signal informing the brain about the level of peripheral energy stores. Infusion of amylin into the cerebral ventricles at 2 pmol/hour for two weeks significantly lowered body weight in rats compared to saline control, and was found to increase energy expenditure and body temperature (45).

Due to the hypoglycemic actions of amylin, the development of a new drug synthetic amylin- pramlintide (Symlin) (also read the related information on the National Diabetes Information Clearinghouse (NDIC) page on NIDDK-NIH website (http://diabetes.niddk.nih.gov/dm/pubs/medicines_ez/insert_L.aspx), has been part of clinical trials to treat diabetes (46-51) and weight loss (52, 53) and has been approved by the U.S. Food and Drug Administration (FDA) for treatment of type I and type II diabetes(54). The treatment of type I diabetes using pramlintide is noteworthy since it has been the only non-insulin analog approved by the FDA for treatment of type I diabetes. The extensive research conducted regarding amylin role in diabetes, and pramlintide's treatment of diabetes has been a driving force in positioning amylin in the spotlight.

5. AMYLIN IS A CARDIOVASCULAR SYSTEM REGULATOR THAT INDUCES ENDOTHELIUM/NITRIC OXIDE (NO)-DEPENDENT OR -INDEPENDENT VASODILATORY EFFECTS, HYPOTENSION AND TACHYCARDIA

Amylin in the bloodstream has been found to decrease blood pressure by 35mm Hg over 30 minutes and have rapid direct vasodilatory effects in rats (55) when given a 100 µg bolus of amylin. These changes were not associated with any epinephrine or norepinephrine changes in plasma, nor was it associated with changes in glucose level (55). Calcitonin gene related peptide (CGRP), also in the calcitonin gene family, causes similar decrease in mean arterial pressure, and has a potency 44 fold compared to amylin (56). To address possible binding to the same receptor, salmon calcitonin (sCT), an amylin agonist that binds to amylin but not CGRP receptors (57), did not result in vasodilation (58) or hypotension (15, 16). This shows that amylin effects in inducing hypotention are due to binding to CGRP receptors.

In one study by Gardiner (59), Long-Evans rats infused with 0.25-2.5nmol/kg/min amylin induced dose dependent tachycardia and hypotension. However, in a separate study by Kaygisiz (60), tachycardia was observed in isolated rat hearts exposed to an amylin antagonist, amylin (8-37). Further, these positive ionotropic effect were found to be completely blocked by diltiazem, a L-type Ca^{2+} channel blocker, and ryanodine, a sarcoplasmic reticulum Ca^{2+} release channel opener (61). This differing response of amylin inducing tachycardia in whole rats *in vivo*, and amylin antagonist inducing tachycardia in isolate rat hearts *ex vivo* is unclear. As discussed previously, amylin induced hypotension is due to actions on the CGRP receptor peripherally. Previous work has also shown that CGRP induced tachycardia is due to reflex activation of the sympathetic nervous system secondary to CGRP induced hypotension (62). Therefore, it is likely that amylin induce bradycardia, and the amylin induced tachycardia observed was most likely due to CGRP mediated hypotension and subsequent reflex tachycardia. A recent report showed that amylin might have been produced vasodilatory, positive chronotropic and positive inotropic effects on rat hearts. Ca^{2+} entry via L-type Ca^{2+} channels, activation of protein kinase C and Ca^{2+} release from SR through ryanodine-sensitive Ca^{2+} channels may be involved in this positive inotropic effect(61).

In addition to amylin effects on heart, amylin has a direct effect on vascular system. For example, amylin (10^{-10}M) induced relaxation of norepinephrine-pre-contracted rat aortic rings by more than 50%. This effect was preserved after blockade of nitric oxide (NO)-synthase and even after denudation of the vessel. Thus amylin-induced vasodilation is an endothelium-independent process not mediated by NO (63). Moreover, amylin also reduced the vascular tone in the isolated, perfused and ventilated rat lung(64). Amylin also induces dose-related vasodilator responses in isolated resistance arteries from the rat mesenteric vascular bed. Vasodilator responses to human CGRP and amylin were not altered after denuding the vascular endothelium, after administration of the nitric oxide synthase inhibitor NG-nitro-L-arginine (L-NA), or after administration of the soluble guanylate cyclase inhibitor 1H-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), suggesting that vasodilator responses to CGRP and Amylin are not mediated by the release of nitric oxide from the vascular endothelium(65). Comparing to the structurally related potent vasodilator neuropeptide calcitonin gene-related peptide, amylin amide was 100 times less. Amylin amide did not induce edema formation; however, as a consequence of its vasodilator activity, amylin amide potentiated edema formation induced in rabbit skin by bradykinin. The intravenous injection of amylin amide (10 nmol) caused a systemic drop in blood

pressure. This study demonstrates that amylin amide elicits vasodilator responses *in vivo*. It is possible that the release of amylin amide from the pancreas in type II diabetes could lead to changes in vascular tone(66). In contrast, a report found that amylin has been linked to the development of hypertension in several pathological states related to hypertension and insulin resistance. Hypertension was higher in a diabetic population carrying the mutation, -132 G/A mutation located within an activator domain of the amylin gene's promoter, than in diabetic noncarriers (74 vs. 57%; $P<0.05$). 384 diabetic carriers showed higher fasting amylin levels than 207 diabetic noncarriers (11.4+/-7 vs. 8.2+/-3 pmol/liter; $P<0.05$). Pre-incubation with 20 pmol/liter amylin impaired the endothelial cell-specific relaxant responses induced by acetylcholine in rat aorta macro-artery and mesenteric microvessels. This endothelial dysfunction-inducing effect was abolished in both vascular beds in the presence of 100 micromol/liter endothelial nitric oxide synthase (eNOS) inhibitor NG-nitro-L-arginine methyl ester(67), suggesting that amyin's endothelial dysfunction-inducing effect is NO-dependent. The future studies are needed to clarify this discrepancy.

In addition, amylin has several other functions somewhat related to vascular biology. Amylin induces renal sodium excretion in sheep by a central angiotensin-dependent mechanism. Amylin may increase renin secretion by a direct effect on the kidney(68). Amylin participates in the maintenance of glucose and calcium homeostasis. Furthermore, amylin inhibits gastric acid secretion. It protects the gastric mucosa in ulcer models like stress, vagal stimulation, ethanol, acetic acid, reserpine and serotonine administration and pylorus ligation(69). Moreover, amylin is also found to be as the disease biomarker in the serum samples of patients with diabetes mellitus(70).

6. AMYLIN IS A NEUROPEPTIDE THAT DEPOLARIZES CHOLINERGIC NEURONS VIA CLOSURE OF SEVERAL DIFFERENT TYPES OF POTASSIUM CHANNELS

In the brain, circulating amylin also activates the circumventricular organs- an area of the brain with an absent blood brain barrier, which is dedicated to transducing the homeostatic signals in the bloodstream (71). In addition to its effects in the bloodstream as a satiety hormone, amylin has also found to be synthesized de novo in neurons throughout the nervous system. Amylin has also been found to cross the blood brain barrier (72, 73). Amylin was also found to enhance memory in rats (74, 75). Its effect on locomotor activity, grooming and rear-earings (standing on hind legs) seem to be mixed. Amylin was found to decrease locomotor activity (76), but increased rearing and grooming behavior (74). Current evidence shows that the role of circulating amylin that crosses the blood brain barrier is involved in long term regulation of homeostasis and not likely to be relevant to the study of brainstem reflexes.

In addition to circulating amylin, amylin has been found to be synthetized *de novo* in the brain. In order to determine if a protein is synthesized within a neuron, *in situ* hybridization can be used to visualize the distribution of mRNAs of the neuropeptide or its receptor. *In situ* hybridization studies with labeled/tagged cRNA or cDNA to amylin mRNA found distribution in small to medium sized nerve cell bodies in dorsal root ganglia from all levels and in the jugular-nodose and trigeminal ganglion (77). Immunohistochemistry studies detecting protein have also found amylin in the trigeminal system within the brainstem (78), and the brain as well as sensory ganglia in the peripheral nervous system (77). It was hypothesized that amylin presence in these various nuclei of the reticular formation may indicate involvement as regulator of some local reflex (78). For instance, various types of alarm or uncomfortable inputs may provoke common responses, including nausea, vomiting, and food refusal. Amylin role in the brainstem is still unclear, and amylin has not been associated with any reflexes. Several studies have been published on the effects of amylin on beta cells in the pancreas, however only one study has examined the effects of amylin on neurons (79). Due to the amyloid forming nature of amylin and Abeta, Jhamandas (79) examined the effects of amylin and Abeta on rat cholinergic basal forebrain neurons under patch-clamp. Neuronal activity of dissociated neurons from the diagonal band of Broca, a cholinergic basal forebrain nucleus was examined. Application of 1 nM to 5 μM of human amylin resulted in a dose-dependent reduction of whole-cell currents in a voltage range from -30 mV to +30 mV (voltage-clamp), reduced Ca^{2+} activated K^+ conductance, and also depressed transient outward and delayed rectifier K^+ currents. These effects were blocked by the amylin receptor antagonist AC187. Diagonal band of Broca contain cholinergic and GABAergic neurons (neurons that produce neurotransmitter gamma-aminobutyric acid (GABA) as their output). However it was found using single cell reverse transcription polymerase chain reaction (RT-PCR) that amylin effects were only seen in cholinergic neurons and not GABAergic neurons. These results suggest that amylin depolarizes cholinergic neurons in the forebrain of rats, and that this depolarization is associated with closure of several different types of potassium channels.

7. CONCLUSION

The presence of amyloid deposits in the pancreas was first described at the beginning of the 20th century. However, it was not until 1987 that the structure of the amylin molecule was identified(80). After 25 years of research, 2398 amylin-related papers and reviews have been published as recorded in the PubMed database. Now it is well accepted that amylin is a circulating multifunctional hormone and neuropeptide that induces vasodilation, depolarizes cholinergic neurons, increases energy expenditure and triggers inflammation. Recent approval of use of amylin analog pramlintide as a new drug by FDA in treating type 1 and 2 diabetes has pushed amylin into the spotlight. Continued improvement of our understanding on this multifunctional hormone would lead to future development of pramlintide as novel therapies for other inflammatory, hematological, metabolic, neurological and vascular diseases.

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