# Amylin and Diabetic Cardiomyopathy – Amylin-Induced Sarcolemmal Ca$^{2+}$ Leak Is Independent of Diabetic Remodeling of Myocardium

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## Abstract

Amylin is a pancreatic β-cell hormone co-secreted with insulin, plays a role in normal glucose homeostasis, and forms amyloid in the pancreatic islets of individuals with type-2 diabetes. Aggregated amylin is also found in blood and extra-pancreatic tissues, including myocardium. Myocardial amylin accumulation is associated with myocyte Ca$^{2+}$ dysregulation in diabetic rats expressing human amylin. Whether deposition of amylin in the heart is a consequence of or a contributor to diabetic cardiomyopathy remains unknown. We used amylin knockout (AKO) mice intravenously infused with either human amylin (i.e, the aggregated form) or non-amyloidogenic (i.e., monomeric) rodent amylin to test the hypothesis that aggregated amylin accumulates in the heart in the absence of diabetes. AKO mice infused with human, but not rodent amylin, showed amylin deposits in the myocardium. Cardiac amylin level was larger in males compared to females. Sarcolemmal Ca$^{2+}$ leak and Ca$^{2+}$ transients were increased in myocytes isolated from males infused with human amylin while no significant changes occurred in either females injected with human amylin or in rat amylin-infused mice. In isolated cardiac myocytes, the amylin receptor antagonist AC-187 did not effectively block the interaction of amylin with the sarcolemma. In conclusion, circulating aggregated amylin accumulates preferentially in male vs. female hearts and its effects on myocyte Ca$^{2+}$ cycling do not require diabetic remodeling of the myocardium.

## Keywords

Diabetic Cardiomyopathy; Amylin; Calcium; Type-2 Diabetes; Prediabetes; Hyperamylinemia

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## 1. Introduction

Type-2 diabetes and insulin resistance are metabolic abnormalities that drive heart failure via multiple mechanisms [1–5]. To compensate for insulin resistance, pancreatic β-cells increase insulin secretion (hyperinsulinemia) [6]. Amylin is a ~4 kDa peptide hormone synthesized and co-secreted with insulin by pancreatic β-cells [7, 8]. Prior studies showed that amylin modulates ingestive behavior [9] and reduces insulin secretion [10–12] and sensitivity [13–16]. Conversely, amylin gene deletion improved glucose tolerance in mice [17] suggesting that amylin plays a role in energy homeostasis.

Amylin from humans aggregates, forming amyloid when overexpressed [8, 18, 19]. Aggregated amylin was shown to cause oxidative stress [20, 21], inflammation [22–24] and apoptosis [18, 25] in the pancreas. In contrast, amylin from species that do not develop type-2 diabetes spontaneously (i.e. mice and rats) has a different amino acid structure and a reduced propensity to form amyloid [26]. Pharmacological induction of insulin resistance in mice expressing human amylin caused amylin amyloid deposition, β-cell apoptosis and overt hyperglycemia [27]. These pathologic changes were replicated in rodents *overexpressing* human [25, 28], but not murine amylin [29]. Thus, hyperamylinemia, is an early contributor to type-2 diabetes.

Accumulating evidence (including our work [30–35]) demonstrates the presence of abundant deposits of aggregated amylin in failing hearts [30–32] from patients with type-2 diabetes or obesity, in brains [33–38] of patients suffering from Alzheimer's disease and in kidneys [39] of patients with type-2 diabetes. The source of amylin deposition in the heart (and brain and kidneys) originates in the pancreas, as no amylin mRNA was found in cardiac [31] and brain [35] tissues. Rats expressing human amylin in the pancreatic β-cells (HIP rats) accumulate aggregated amylin in the pancreas [29], heart [30], brain [34, 40] and kidneys [41], similar to humans with type-2 diabetes [30–39]. HIP rats develop type-2 diabetes [29] and heart dysfunction characterized by diastolic dysfunction [30], cardiac hypertrophy [30, 31] and dilation [31]. At the myocyte level, we found increased cytosolic $Ca^{2+}$ and sarcolemmal $Ca^{2+}$ leak in HIP rats, but not in glucose- and age-matched diabetic rats that express endogenous rat amylin [30]. We also reported that mitigating myocardial accumulation of amylin improves myocyte $Ca^{2+}$ handling and heart function in diabetic HIP rats [31]. However, it is unclear whether cardiac accumulation of amylin is promoted by diabetic remodeling of the heart or would arise independent of such remodeling.

In HIP rat hearts, we found that aggregated amylin accumulates both interstitially [32] and inside myocytes [31, 32]. A potential venue for the amylin uptake in cardiac myocytes may involve binding of amylin monomers to calcitonin gene-related peptide (CGRP) receptors. CGRP receptors appear to play a role in internalization of monomeric amylin in pancreatic β-cells [43] and neurons [44] and they are expressed in cardiac myocytes [42]. Alternatively, cardiac amylin accumulation may result from deposition of aggregated amylin circulating in the blood. This mechanism is consistent with our previously published data [32] showing that amylin aggregates detected in cardiac myocytes, pancreas and blood all have similar molecular weights.

Here, we tested the hypothesis that the cardiac buildup of amylin and its subsequent effects on myocyte function are independent of diabetes-mediated remodeling of the myocardium. To test this hypothesis, we measured cardiac amylin accumulation and myocyte $Ca^{2+}$ cycling in amylin knockout (AKO) mice intravenously infused with either aggregated human amylin or non-amyloidogenic rat amylin. The reason for using AKO mice, not simply WT mice, for this study, is twofold. First, it eliminates the potential confound factor that rodent amylin and human amylin may form mixed oligomers. Second, comparing myocytes from WT vs. AKO mice will clarify whether lack of amylin affects myocyte $Ca^{2+}$ cycling. Of note, previous investigations [17] demonstrated increased insulin secretion and improved glucose tolerance in AKO mice compared to WT littermates. Blood glucose elimination was enhanced in female compared to male AKO mice. Thus, lack of amylin appears to improve insulin secretion and glucose homeostasis in mice. Using isolated cardiac myocytes, we also investigated the efficacy of the CGRP receptor antagonist AC-187 to prevent the myocyte amylin uptake and downstream cytotoxicity. Our findings may contribute to the understanding of the complex molecular mechanisms underlying diabetic cardiomyopathy.

## 2. Materials and methods

### 2.1. Experimental animals

Animal studies conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committees at University of Kentucky. Frozen embryos of amylin knockout mice (AKO mice; line B6.129P2-Iapp^tm1Sgm/Kctt) were purchased from EMMA (https://www.infrafrontier.eu/) and re-derived at Jackson Laboratory. Genotyping was performed as described in Ref [17]. A total of 55 AKO mice were used in the study (n=32 males and n=23 females), at an age of 8–9 months. AKO mice were infused with either aggregated human amylin (n=9 for each sex) or non-amyloidogenic rat amylin (n=5 per group/per sex) and used for measurements of myocyte amylin deposition, $Ca^{2+}$ transients and sarcolemmal $Ca^{2+}$ leak. N=9 AKO mice of both sexes were used as controls. Additionally, n=9 male AKO mice were used for assessing amylin accumulation in the myocardial tissue upon infusion of human or rat amylin (vs. control; 3 mice per group). WT littermate mice (n=4 males and n=4 females) were used to measure the baseline $Ca^{2+}$ transient amplitude in cardiac myocytes and plasma interleukin (IL)-1β. Metabolic characterization of AKO vs. WT mice was previously published [17].

#### Intravenous infusion of amylin

AKO mice were intravenously infused, via the tail vein, with either aggregated human amylin (2 μg/g body weight; AS-60804, AnaSpec, CA) or rat amylin (2 μg/g body weight; 74-5-10, American Peptide, CA) for 7 days (q.d.). For inducing aggregation, human was dissolved in PBS (pH 7.4) at a concentration of 50 μM and maintained at 37°C for 72 hours with occasional shaking [34]. Our previous data [34] show that blood from AKO rats infused with human amylin under similar conditions (2 μg/g body weight, q.d., 7 days) has comparable distributions of amylin-positive molecular complexes with blood from HIP rats and humans with type-2 diabetes.

### Cardiac myocyte isolation

The experimental protocol was previously [30, 31] described. Briefly, mice were anesthetized by isoflurane (4.0%). When deep anesthesia was reached, hearts were excised quickly, placed on a Langendorff perfusion apparatus and perfused with buffer containing 1 mg/ml collagenase. When the heart became flaccid, the left ventricular tissue was cut into small pieces, dispersed, filtered and kept in a standard Tyrode's solution containing (in mmol/L): 140 NaCl, 4 KCl, 1 $MgCl_2$, 10 glucose, 5 HEPES, and 1 $CaCl_2$ (pH=7.4). All experiments were performed at room temperature.

### Amylin accumulation in cardiac myocytes

Isolated cardiac myocytes were lysed as described before [32] and amylin content was assessed by ELISA (EIA-AM, Raybiotech, GA) according with manufacturer's protocol.

### Immunofluorescence

Immunofluorescence was used in myocardial tissue and isolated myocytes from AKO mice infused with aggregated human amylin and control AKO mice (no amylin infusion). The primary antibodies were anti-human amylin antibody (1:200, SC-377530, Santa Cruz, TX; raised in rabbit) and CGRP antibody (ab47027, abcam, Cambridge, MA; raised in rabbit). The secondary antibody were Alexa Fluor 568 conjugated anti-rabbit IgG (A11036, Invitrogen, MA) and Texas red conjugated anti-rabbit IgG (SC-2780; Santa Cruz; TX).

### $Ca^{2+}$ transient measurements

$Ca^{2+}$ transients were measured in isolated myocytes loaded with Fluo-4 (10 μM for 20 min) and electrically stimulated at 0.2, 0.5, 1 and 2 Hz.

### Sarcolemmal $Ca^{2+}$ leak

To measure the passive $Ca^{2+}$ leak across sarcolemma, myocytes were loaded with Fura-2 (10 μM for 25 min) and pre-incubated (10 min) with 10 μM thapsigargin to block SERCA and empty the SR of $Ca^{2+}$. Sarcolemmal $Ca^{2+}$ leak was then measured the initial rate of $[Ca^{2+}]_i$ decline upon reducing $[Ca^{2+}]_o$ from 1 mM to 0, with the $Na^+/Ca^{2+}$ exchanger blocked by using a $0Na^+/0Ca^{2+}$ external solution ($Na^+$ was replaced by $Li^+$, $CaCl_2$ was omitted and 10 mM EGTA added to the Tyrode's solution) and the plasma membrane $Ca^{2+}$ ATPase inhibited with carboxyeosin (20 μM), as before [30].

### IL-1β measurement

Plasma IL-1β was measured by ELISA (RAB0277, Sigma, MO, US) according with manufacturer's protocol.

### Statistical analysis

Comparison of two groups was done using unpaired Student's t-test. One-way analysis of variance with Bonferroni's post hoc test was used to compare multiple groups. Data are presented as means ± SEM. Difference between groups was considered significant when $P < 0.05$. All analyses were performed using GraphPad Prism 5.0 software.

## 3. Results

### 3.1. Lack of basal amylin has no effect on myocyte Ca²⁺ cycling

We have previously [30] shown that incubation of isolated cardiac myocytes with human amylin (50 μM; 2 hour incubation time) results in a significant increase of the $Ca^{2+}$ transient amplitude [30]. A modest increase of $Ca^{2+}$ transient amplitude was also measured in cardiac myocytes incubated with rat amylin 50 μM; 2 hour incubation time). To test whether lack of basal amylin expression alters myocyte $Ca^{2+}$ handling, we compared $Ca^{2+}$ transients in cardiac myocytes isolated from WT and AKO mice (both males and females; Fig 1). Our results (Fig 1) showed similar amplitude and decline time for $Ca^{2+}$ transients in myocytes from WT and AKO mice. So, the presence of endogenous mouse amylin had no effect on basal myocyte $Ca^{2+}$ cycling.

### 3.2. Circulating aggregated amylin accumulates in the myocardium in absence of diabetes

Male and female AKO mice were intravenously infused with either aggregated human amylin or non-amyloidogenic (i.e, monomeric) rat amylin (2 μg/g body weight) for 7 days (q.d.), as described in Methods. Mice were then euthanized for cardiac myocyte isolation. The level of amylin present in myocyte lysates from AKO mice infused with aggregated human amylin (h-Amy) or rodent amylin (r-Amy) were measured by ELISA (Fig 2A). Myocytes from males injected with aggregated human amylin show significant accumulation (P=0.02, One-sample t-test to compare the mean to zero) whereas no amylin was found in myocytes from females injected with human amylin (P=0.45). Infusion of rat amylin resulted in no significant myocardial amylin accumulation in either male (P=0.96) or female (P=0.64) mice.

The presence of amylin in myocyte lysates from male AKO mice infused with aggregated human amylin indicates that amylin attached to the sarcolemma and/or incorporated into cardiac myocytes. To further test the attachment of circulating aggregated amylin to the sarcolemma from, we labeled isolated myocytes with an anti-amylin antibody and performed immunofluorescence (Fig 2B). Myocytes from human amylin-infused AKO male mice showed a strong immunofluorescence signal at the sarcolemma, consistent with the ELISA results in Fig 2A.

Fig 2C–E displays the results of immunofluorescent staining of amylin (green) in myocardial sections from AKO mice with or without amylin injection. Myocardial tissue from male AKO mice infused with aggregated human amylin showed a robust amylin immunofluorescence signal within the interstitial spaces or/and at the sarcolemma (Fig 2C). In contrast, immunofluorescence staining of myocardial tissues from male AKO mice infused with rodent amylin (Fig 2D) is similar to that in controls (AKO mice with no amylin infusion; Fig 2E), indicating the lack of amylin deposition in myocardium. In these experiments, HIP rat pancreatic tissue served as positive control for amylin deposition (Fig 2F).

Thus, acute intravenous infusion of aggregated human amylin (but not rodent amylin) caused myocardial amylin deposition and attachment to the sarcolemma in male AKO mice.

Present results are consistent with our previous data from HIP rats [30–32] and isolated cardiac myocytes incubated with exogenous human amylin [30, 32].

### 3.3. Myocardial amylin accumulation alters myocyte $Ca^{2+}$ cycling

Next, we tested whether circulating amylin affects myocyte $Ca^{2+}$ cycling by measuring $Ca^{2+}$ transients in myocytes from male and female AKO mice infused with either aggregated human amylin or rat amylin versus control AKO mice (Fig 3). At all stimulation frequencies, $Ca^{2+}$ transient amplitude was significantly larger in myocytes from male AKO mice infused with aggregated human amylin versus control AKO male (Fig 3A,C). In contrast, infusion of rat amylin in male AKO mice induced no effect on myocyte $Ca^{2+}$ cycling (Fig 3A,C), in agreement with the lack of myocardial amylin deposition in this group (Fig 2A). Cardiac myocytes from AKO females infused with human or rat amylin, which did not accumulate amylin (Fig 2A), showed no change in $Ca^{2+}$ transient amplitudes (Fig 3B,D). Neither human or rat amylin-infusion altered the decline of $Ca^{2+}$ transients, consistent with our prior data in isolated myocytes incubated with amylin [34]. Thus, myocardial amylin accumulation is the cause for the larger $Ca^{2+}$ transient amplitude in male AKO mice infused with aggregated human amylin, consistent with our previous results from HIP rats [30] and isolated cardiac myocytes incubated with exogenous human amylin [30].

### 3.4. Circulating aggregated amylin provokes sarcolemmal $Ca^{2+}$ leak

We hypothesized that myocardial amylin accumulation alters myocyte $Ca^{2+}$ cycling by increasing sarcolemmal $Ca^{2+}$ permeability. To test this hypothesis, we measured the passive sarcolemmal $Ca^{2+}$ leak in myocytes from male AKO mice intravenously infused with aggregated human amylin control AKO mice (no amylin infusion). Myocytes were pre-incubated with thapsigargin to inhibit SERCA and thus empty the sarcoplasmic reticulum of $Ca^{2+}$. Resting $[Ca^{2+}]_i$ under these conditions was similar in myocytes from human amylin-infused and control AKO males (Fura-2 ratio of 2.63±0.10 *vs.* 2.57±0.10). We then measured the initial rate of $[Ca^{2+}]_i$ decline upon reducing $[Ca^{2+}]_0$ from 1 to 0 mmol/L with $Na^+/Ca^+$ exchanger, $Ca^{2+}$ influx and plasma membrane $Ca^{2+}$-ATPase blocked (Fig 4A). Sarcolemmal $Ca^{2+}$ leak was significantly larger in myocytes from human amylin-infused rats compared to controls (Fig 4B).

### 3.5. Blocking CGRP receptors does not mitigate the amylin-mediated effects on myocytes, ex vivo

Previous work [42] suggested that amylin binds to the CGRP receptors on the sarcolemma. We tested whether blocking CGRP receptors by the AC-187 inhibitor reduces the interaction of amylin with cardiac myocytes, *ex vivo*. Immunoconfocal microscopy showed co-localization of amylin with CGRP receptors at the sarcolemma in cardiac myocytes incubated with human amylin (100 nM for 6 h; Fig 5A). However, co-incubation of isolated myocytes with the CGRP receptor AC-187 (100 nM) did not reduce the human amylin-provoked sarcolemmal $Ca^{2+}$ leak (Fig 5B). In contrast the membrane sealant P188 prevented the increase in sarcolemmal $Ca^{2+}$ leak in myocytes incubated with aggregated amylin (Fig 5B), which is consistent with our previous data [32] indicating that P188 efficiently reduces amylin-mediated peroxidative damage of the sarcolemma.

### 3.6. Circulating aggregated amylin triggers systemic inflammation in male AKO mice

Accumulation of aggregated amylin in cells and tissues (including the heart) triggers a proinflammatory response [22–24, 32]. The plasma level of the proinflammatory cytokine IL-1β was elevated in male AKO mice infused with aggregated amylin (Fig 6). This systemic inflammatory response to aggregated amylin was not observed in female AKO mice. Infusion of rat amylin had no effect on plasma IL-1β in AKO mice (Fig 6).

## 4. Discussion

In summary, we found that intravenous infusion of aggregated human amylin leads to amylin accumulation in the myocardium and an increase in sarcolemmal $Ca^{2+}$ leak and $Ca^{2+}$ transient amplitudes in healthy, non-diabetic AKO mice. Alteration of sarcolemmal $Ca^{2+}$ fluxes by circulating aggregated amylin in AKO mice is consistent with our previously published data [32] showing sarcolemmal peroxidative injury and cardiac inflammation in normal WT mice infused with aggregated amylin. These results suggest the following: *i*) the presence of amylin aggregates in hearts of humans with type-2 diabetes [30–32] is the result of higher levels of circulating aggregated amylin in the pre-diabetic state (when the secretion of insulin, and thus amylin, is greatly increased) rather than as a consequence of cardiac diabetic remodeling or heart failure; *ii*) human hearts undergoing the amylin stress are likely predisposed to a more rapid destabilization of sarcolemmal processes in diabetes.

In contrast to human (amyloidogenic) amylin, intravenous injection of monomeric rat amylin did not result in cardiac amylin deposition and had no effects on myocyte $Ca^{2+}$ cycling. These results are consistent with previous studies [8, 18–22, 25–32] showing that amylin accumulation in cells and tissues is promoted by the amyloidogenicity of human amylin and increased propensity of aggregated amylin to incorporate in cellular membranes. Myocardial amylin accumulation may also be explained by an impaired clearance of aggregated amylin. Future studies need to investigate factors contributing to the impaired clearance of circulating aggregated amylin *in vivo*.

Blood glucose elimination is enhanced in female AKO mice compared to males [17]. We found that aggregated human amylin incorporated only in hearts from males but not female AKO mice, indicating a significant sex-dependent effect which might be estrogen-dependent. Indeed, we recently [34] showed that, compared to males, female HIP rats developed hyperglycemia and neurologic deficits later in life (i.e., ~12 months vs. ~18 months of age). These results are consistent with previous data [45] indicating that pancreatic amylin deposition is more abundant in men compared to women, which was attributed to increased insulin resistance in men [46, 47]. Ovariectomy or employing older female mice (as in our recent study in HIP rats [34]) might mitigate the sex difference in amylin-induced pathology. Indeed, previous studies demonstrated sex differences in cardiac myocyte ion channels [48–52], $Ca^{2+}$ cycling [53, 54], contractions [53] and metabolism [55, 56], which may contribute to the differential effects of amylin stress. Elucidating sex-specific cardiac responses to amylin stress may help in understanding the development and progression of heart failure in men vs. women suffering from diabetes.

Previously published data showed that inducing diabetic states alters myocyte $Ca^{2+}$ cycling [1, 57–59] in rodents, even in the absence of cardiac amylin deposition. The proposed mechanism for diabetes-induced $Ca^{2+}$ dysregulation (see Fig 7; schematic mechanism) involves activation of CaMKII due to O-linked glycosylation (magenta pathway) [60] and oxidation (orange pathway) [61] of the kinase. CaMKII activation enhances sarcoplasmic reticulum (SR) $Ca^{2+}$ leak through phosphorylation of ryanodine receptors (RyRs). Moreover, oxidative stress favors oxidation of the thiol residues of RyRs, which increases the opening probability of the channels [62]. Thus, the induction of diabetic states in rodents without cardiac amylin accumulation affects myocyte $Ca^{2+}$ cycling mainly via the SR $Ca^{2+}$ leak. Our results indicate that cardiac amylin accumulation alters myocyte $Ca^{2+}$ cycling through provoking sarcolemmal $Ca^{2+}$ leak (Fig 7; brown pathway), an effect that does not entail diabetic remodeling of myocardium. Our previous results [30, 31] showed that myocyte $Ca^{2+}$ dysregulation caused by the enhanced sarcolemmal $Ca^{2+}$ leak activates $Ca^{2+}$-mediated transcriptional signaling leading to SERCA downregulation. Thus, amylin-induced sarcolemmal $Ca^{2+}$ leak appears as a new and complementary mechanism of cardiac $Ca^{2+}$ dysregulation in diabetic cardiomyopathy. While our previous study [30] showed that cardiac amylin accumulation is associated with diastolic dysfunction in HIP rats, the functional *in vivo* consequences of the infusion of aggregated amylin in AKO mice have not been explored, which is a limitation of the present work.

In pancreatic β-cells [43] and neurons [44], binding of amylin to CGRP receptors is an important mechanism of internalization of monomeric amylin. Blocking CGRP receptors with the AC-187 inhibitor did not reduce amylin-mediated sarcolemmal $Ca^{2+}$ leak in isolated cardiac myocytes suggesting that other mechanisms contribute to amylin-mediated myocyte $Ca^{2+}$ dysregulation, i.e., sarcolemmal injury. Other possible explanations are that the 100 nM amylin concentration overcomes the CGRP receptor blockade or/and a lower selectivity of the AC-187 inhibitor for cardiac myocyte CGRP receptors compared to neurons and β-cells.

## 5. Conclusions

Cardiac amylin accumulation and subsequent amylin-mediated effects on myocyte $Ca^{2+}$ cycling result from circulating aggregated (human) amylin, but not monomeric (rat) amylin and do not entail pre-existing diabetic remodeling of myocardium or heart failure. Lack of basal amylin has no effect on cardiac contractility; however, circulating aggregated amylin provokes myocyte $Ca^{2+}$ mishandling by enhancing the sarcolemmal $Ca^{2+}$ leak.

Hyperamylinemia is common in prediabetes [6–8] and promotes amylin aggregation [8, 25, 30, 31]. Aggregated amylin was identified in the blood [32] and cardiac tissue [30] of patients with type-2 diabetes or obesity. Present results demonstrate that circulating aggregated amylin induces cardiotoxicity independent of diabetic remodeling of myocardium. Thus, circulating aggregated amylin represents a potential therapeutic target in diabetic cardiomyopathy.

## Acknowledgments

## Abbreviations

| | |
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| **AKO mice** | amylin knockout mice |
| **CGRP** | calcitonin gene-related peptide |
| **HIP rats** | rats expressing the human islet amyloid polypeptide (IAPP; amylin) |
| **WT mice** | wild-type mice |
| **h-Amy** | human amylin |
| **r-Amy** | rat amylin |

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**Highlights**

- Basal amylin has no effect on myocyte $Ca^{2+}$ cycling.

- Aggregated amylin provokes myocyte $Ca^{2+}$ dysregulation by enhancing sarcolemmal $Ca^{2+}$ leak.

- Myocyte uptake of amylin does not entail pre-existing diabetic remodeling of myocardium.

- Circulating aggregated amylin, but not monomeric amylin, accumulates in myocardium.

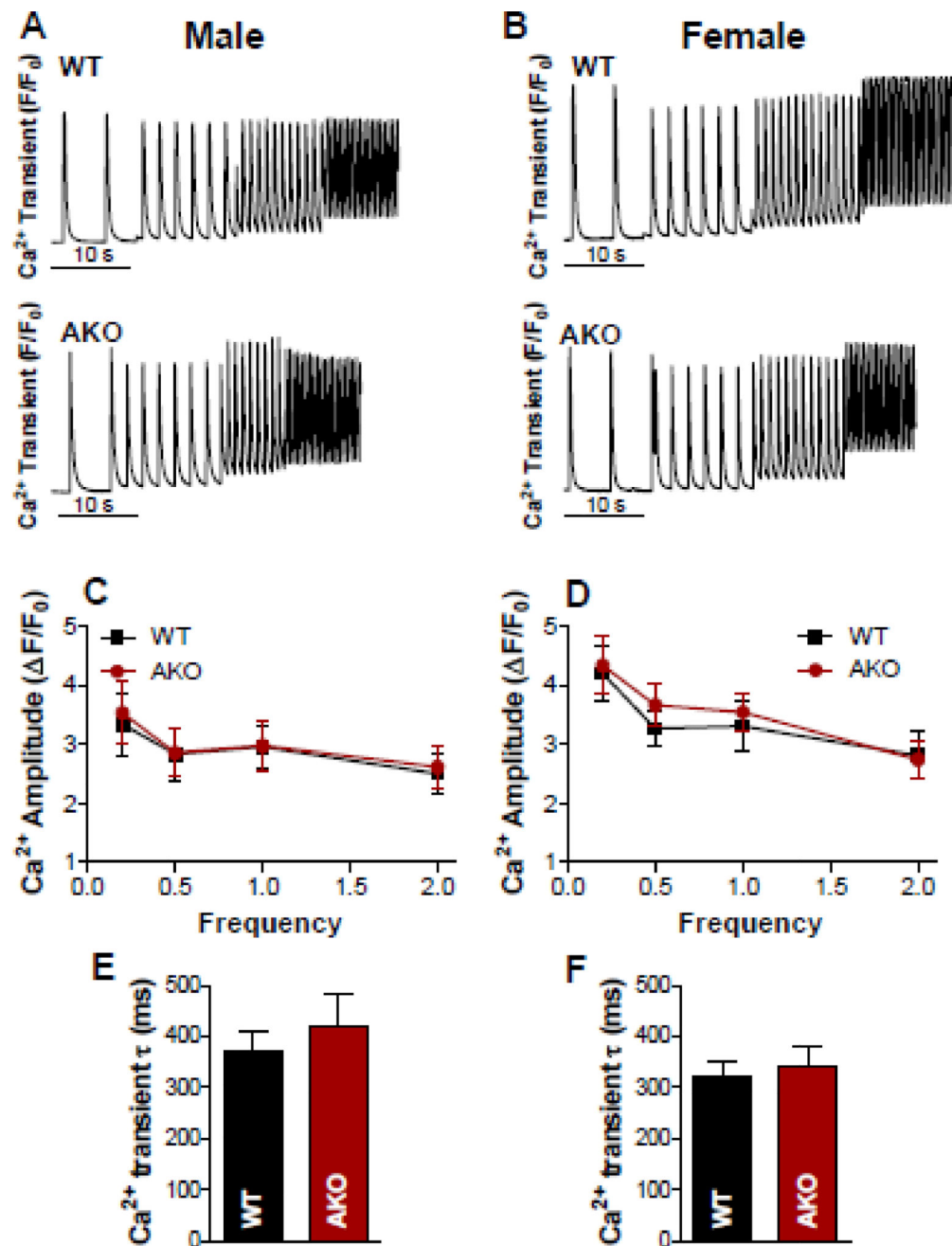- Cardiac amylin accumulation is larger in male compared to female mice.

**Figure 1. Basal amylin has no effect on myocyte Ca$^{2+}$ cycling**

(A–B) Representative recordings of Ca$^{2+}$ transients triggered at 0.2, 0.5, 1 and 2 Hz in cardiac myocytes from male (A) and female (B) WT and AKO mice. (C) Ca$^{2+}$ transient amplitudes in cardiac myocytes from male WT mice (10 cells, 4 mice) vs. male AKO mice (11 myocytes, 4 mice). (D) Ca$^{2+}$ transient amplitudes in cardiac myocytes from female WT mice (11 cells, 4 mice) vs. female AKO mice (13 myocytes, 3 mice). (E-F). The decline time of Ca$^{2+}$ transients triggered by excitation at 0.5 Hz in myocytes from male (C) and female (D) WT and AKO mice. In all groups, data were recorded from 2–3 myocytes/mouse and are presented as the mean±SEM for myocytes.
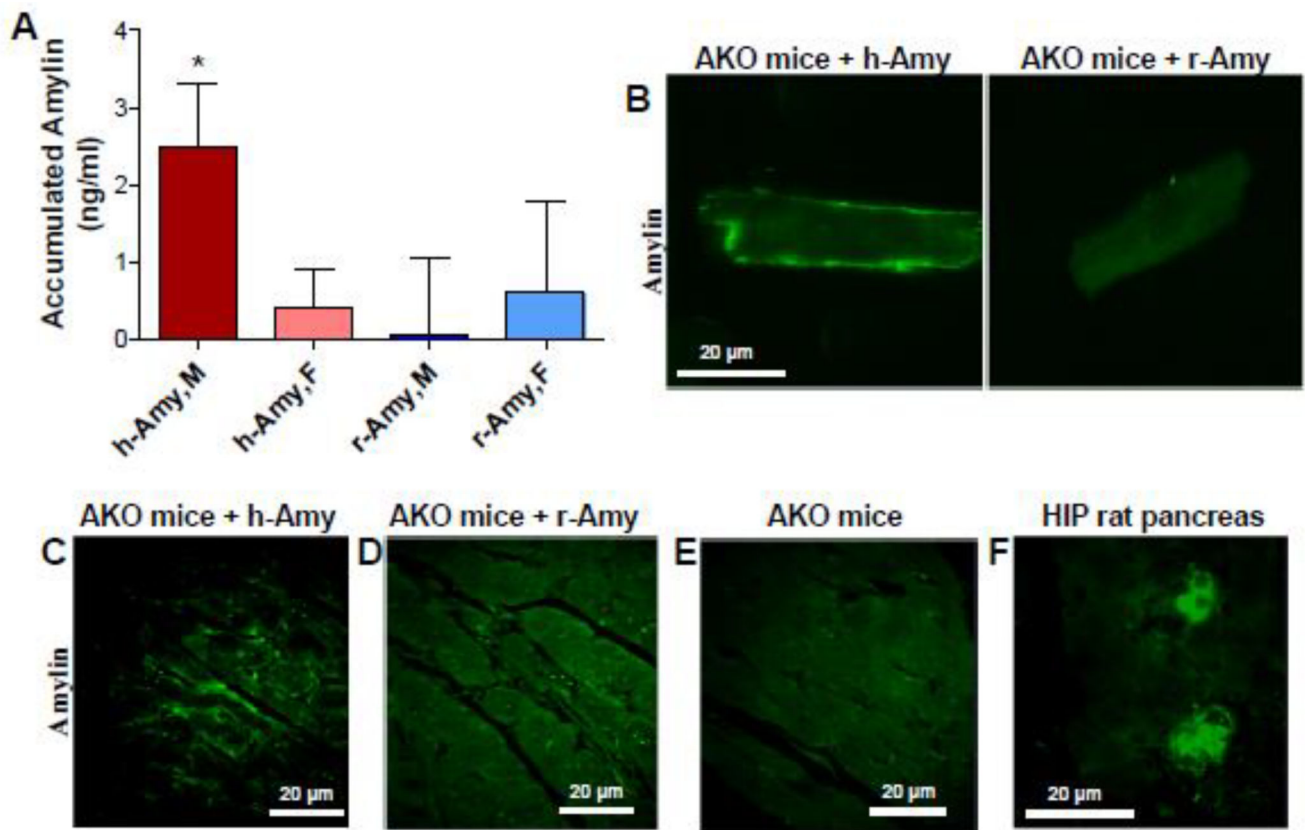
**Figure 2. Circulating aggregated (human) amylin, but not monomeric (rat) amylin, promotes myocardial amylin accumulation**

(A). ELISA data showing the average levels of amylin present in cardiac myocyte lysates from male and female AKO mice infused with either aggregated human amylin (h-Amy) or rat amylin (r-Amy). Measurements were done in 9 males and 5 females for both h-Amy and r-Amy. *P<0.05 using One-sample t-test to compare the mean to zero). (B). Immunofluorescence staining of amylin in cardiac myocytes isolated from amylin infused-male AKO mice vs. male AKO mice infused with rat amylin (r-Amy). (C–E). Immunofluorescence staining of amylin in cardiac sections from amylin infused-male AKO mice vs. control AKO mice (no amylin infusion). (F). Immunofluorescence staining of amylin in pancreatic tissue from HIP rats (positive control for amylin staining).
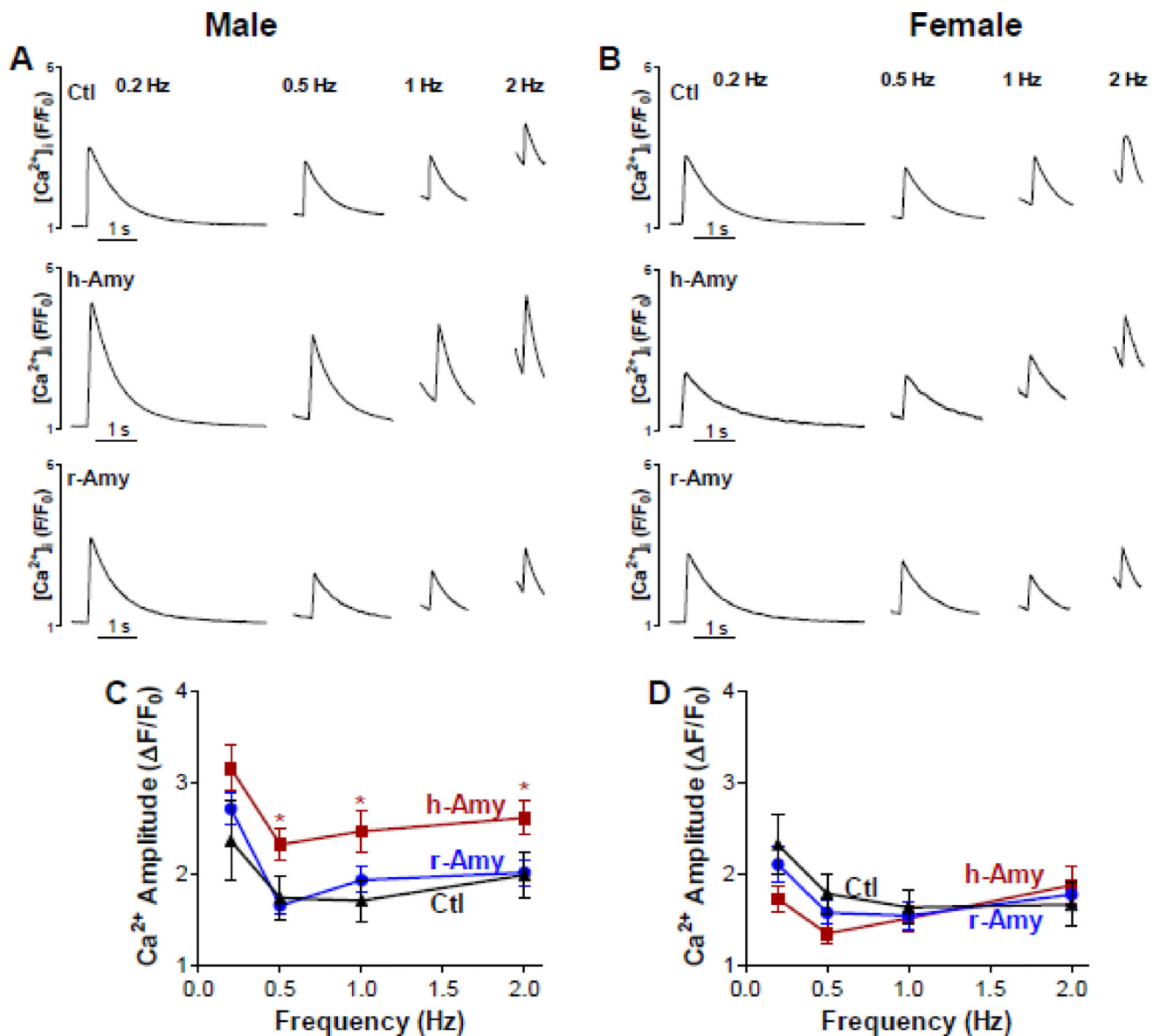
**Figure 3. Circulating aggregated amylin increases myocyte Ca$^{2+}$ transient amplitude**

(A–B) Ca$^{2+}$ transients recorded in myocytes from control AKO mice, AKO mice that were infused with aggregated human amylin (h-Amy) and AKO mice injected with monomeric rat amylin (r-Amy). Panel (A) shows recording in myocytes from males and panel (B) shows myocytes from females. (C) Mean Ca$^{2+}$ transient amplitude in cardiac myocytes from male AKO mice infused with either aggregated human amylin (24 cells, 6 mice) or rat amylin (35 myocytes from 6 mice), and control AKO males (15 cells, 3 mice). (D) Mean Ca$^{2+}$ transient amplitude in cardiac myocytes from female AKO mice infused with aggregated human amylin (29 cells, 6 mice) or rat amylin (32 cells, 6 mice) and control AKO females (14 cells, 3 mice).
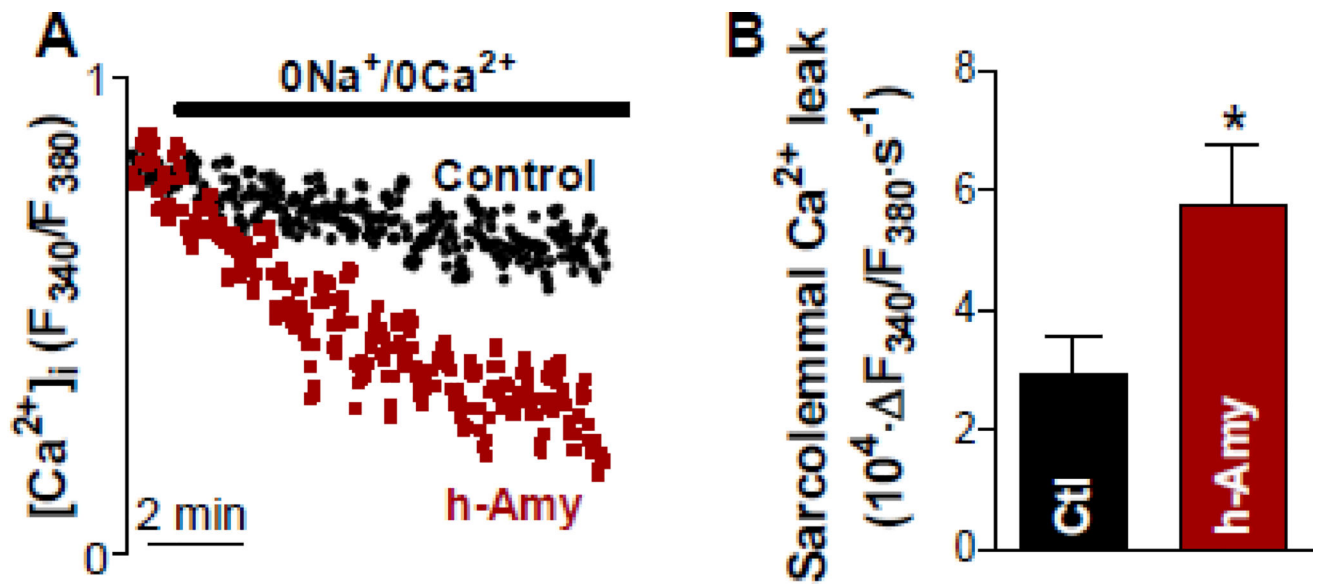
**Figure 4. Circulating aggregated amylin provokes sarcolemmal $Ca^{2+}$ leak**
(A) Representative examples of passive sarcolemmal $Ca^{2+}$ leak measurements. (B) Mean data for the sarcolemmal $Ca^{2+}$ leak in cardiac myocytes from male AKO mice infused with aggregated human amylin (h-Amy; 15 myocytes, 3 rats) and from male AKO mice with no amylin infusion (control; 12 myocytes, 3 mice). * $P<0.05$, Student's t-test
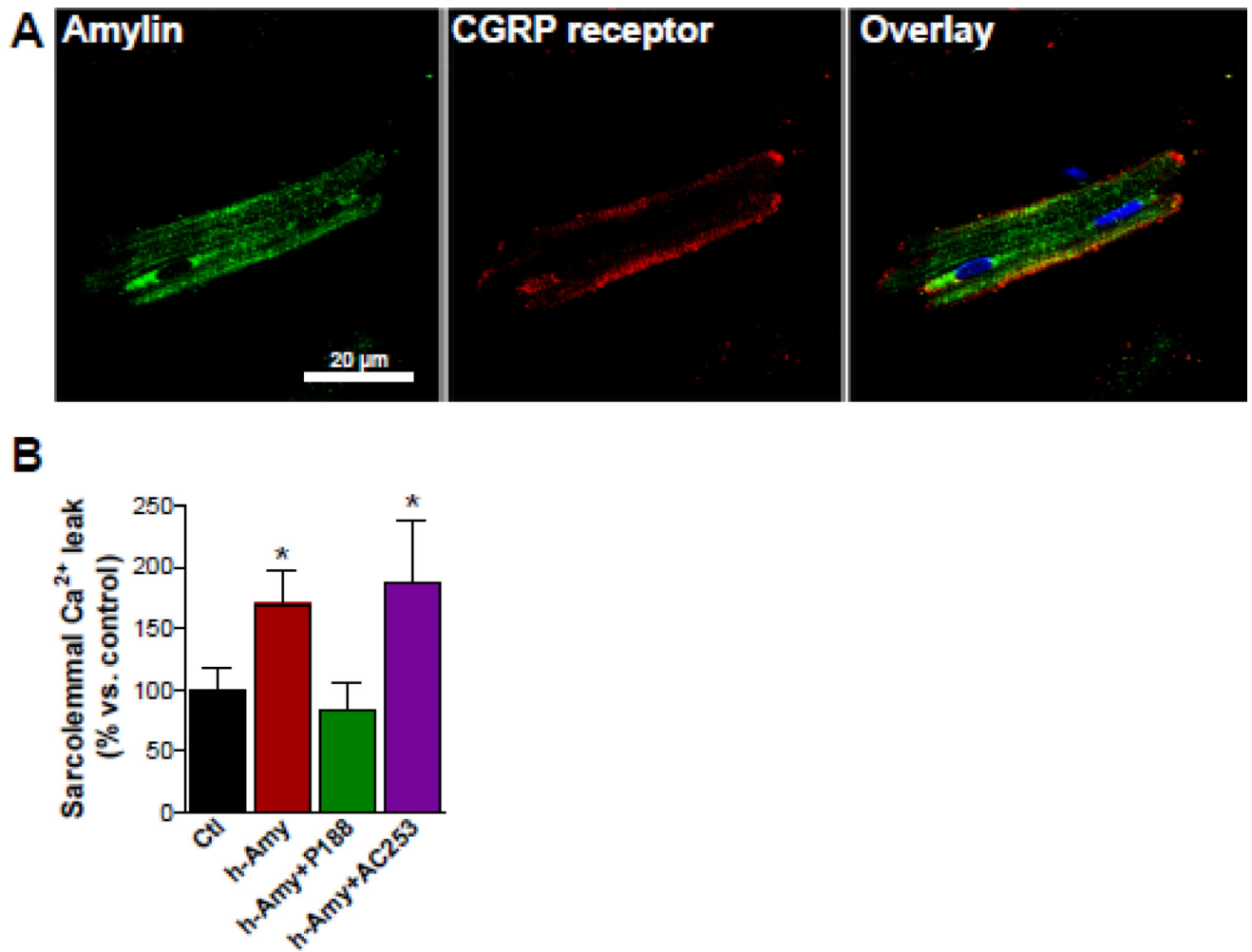
**Figure 5. Inhibition of CGRP receptors has no significant effect on the ex vivo attachment of amylin to cardiac myocytes**

(A) Dual immunofluorescence staining of amylin (green) and CGRP receptor (red) in cardiac myocytes incubated ex vivo with human amylin (100 nM for 6 hours). (B) Passive sarcolemmal $Ca^{2+}$ leak in cardiac myocytes from male AKO mice co-incubated with human amylin (100 nM) for 6 h and either the AC-187 inhibitor (100 nM) or the P188 membrane sealant (100 nM). Cardiac myocytes from male AKO mice (no amylin) were used as controls.For each group, measurements were done on   6 myocytes from 3 different mice. *P<0.05.
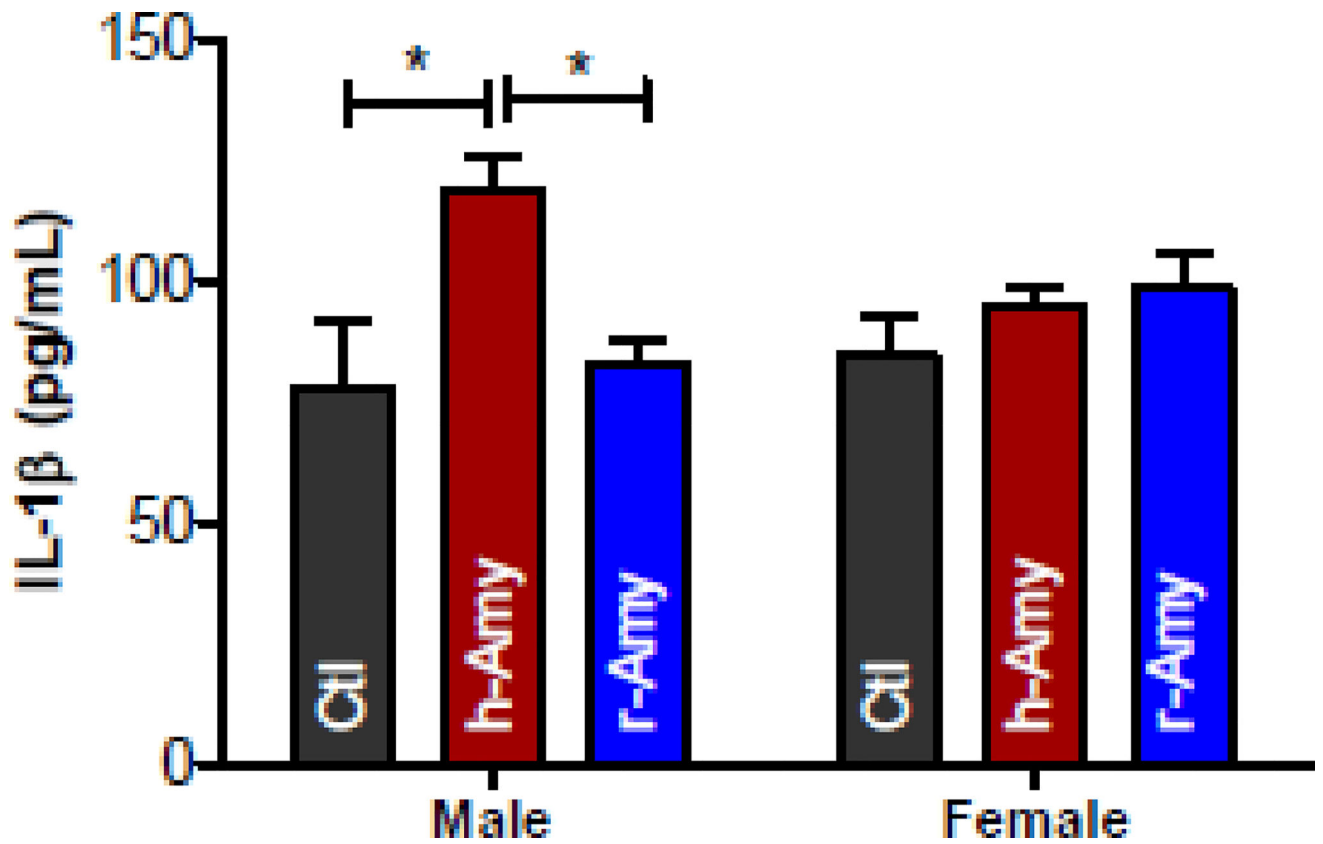
**Figure 6. Circulating aggregated amylin triggers systemic inflammation in male AKO mice**
ELISA data showing the average levels of IL-1β in plasma from male and female AKO mice infused with either aggregated human amylin (h-Amy) or rat amylin (r-Amy). Measurements were done in 9 males and 5 females for both h-Amy and r-Amy. Plasma from WT littermate mice (n=4 males and n=4 females) was used as control. *P<0.05 using One-sample t-test to compare the mean to zero).
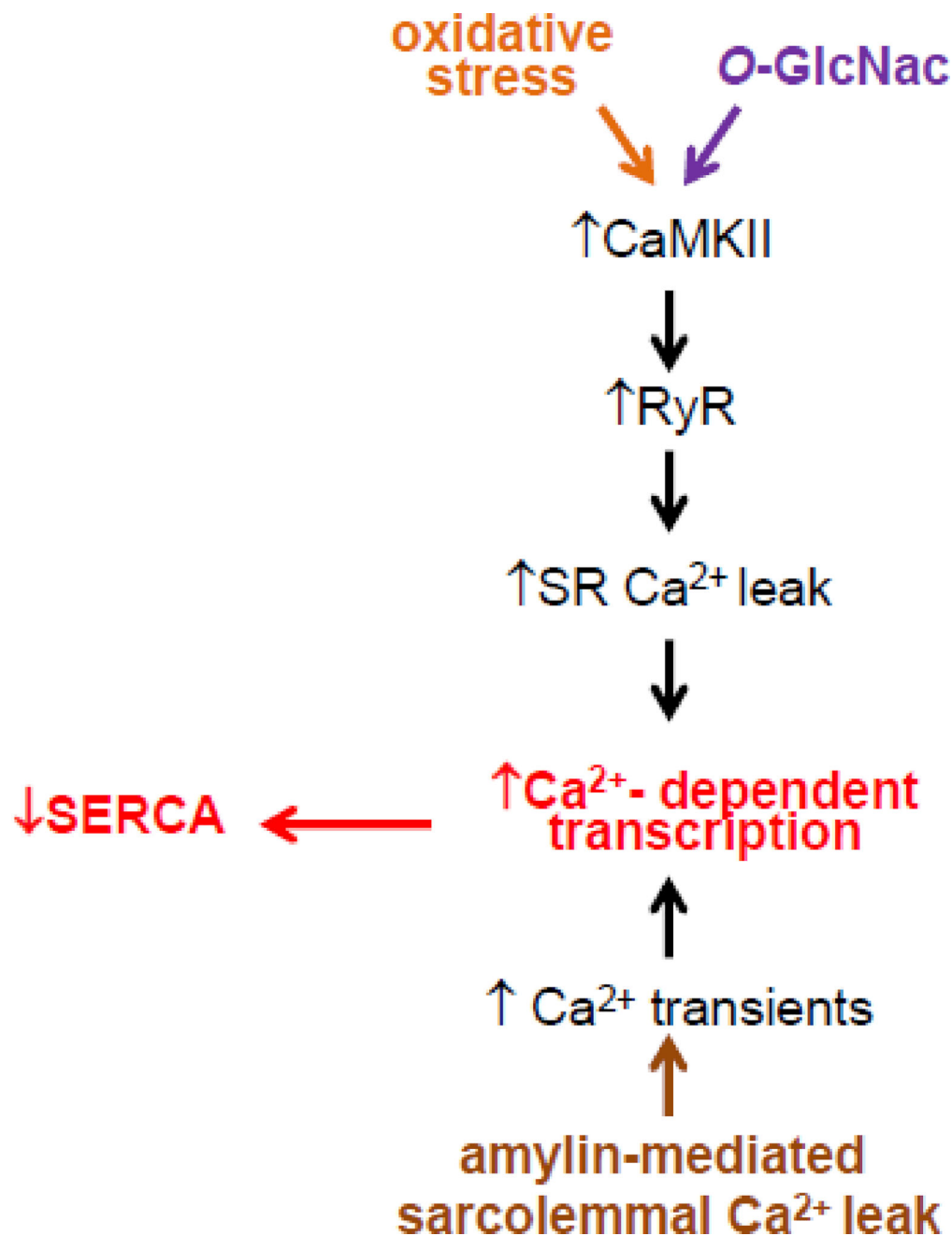
**Figure 7. Proposed mechanism for the additive effect of amylin-induced sarcolemmal Ca$^{2+}$ leak to myocyte Ca$^{2+}$ dysregulation in diabetes**

Diabetes-induced Ca$^{2+}$ dysregulation involves activation of CaMKII due to O-linked glycosylation (magenta pathway) [60] and oxidation (orange pathway) [61] of the kinase. CaMKII activation enhances sarcoplasmic reticulum (SR) Ca$^{2+}$ leak through phosphorylation of ryanodine receptors (RyRs). Thus, the induction of diabetic states in rodents without cardiac amylin accumulation affects myocyte Ca$^{2+}$ cycling mainly via the SR Ca$^{2+}$ leak. Cardiac amylin accumulation alters myocyte Ca$^{2+}$ cycling through provoking

sarcolemmal $Ca^{2+}$ leak (brown pathway), an effect that does not entail diabetic remodeling of myocardium.