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Cardiovascular effects of exogenous adrenomedullin and CGRP in *Ramp* and *Calcrl* deficient mice

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Abstract

Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) are potent vasodilator peptides and serve as ligands for the G-protein coupled receptor (GPCR) calcitonin receptor-like receptor (CLR/*Calcrl*). Three GPCR accessory proteins called receptor activity-modifying proteins (RAMPs) modify the ligand binding affinity of the receptor such that the CLR/RAMP1 heterodimer preferably binds CGRP, while CLR/RAMP2 and CLR/RAMP3 have a stronger affinity for AM. Here we determine the contribution of each of the three RAMPs to blood pressure control in response to exogenous AM and CGRP by measuring the blood pressure of mice with genetic reduction or deletion of the receptor components. Thus, the cardiovascular response of *Ramp1*$^{-/-}$, *Ramp2*$^{+/-}$, *Ramp3*$^{-/-}$, *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ double-knockout (dKO), and *Calcrl*$^{+/-}$ mice to AM and CGRP were compared to wildtype mice. While under anesthesia, *Ramp1*$^{-/-}$ male mice had significantly higher basal blood pressure than wildtype males; a difference which was not present in female mice. Additionally, anesthetized *Ramp1*$^{-/-}$, *Ramp3*$^{-/-}$, and *Calcrl*$^{+/-}$ male mice exhibited significantly higher basal blood pressure than females of the same genotype. The hypotensive response to intravenously injected AM was greatly attenuated in *Ramp1*$^{-/-}$ mice, and to a lesser extent in *Ramp3*$^{-/-}$ and *Calcrl*$^{+/-}$ mice. However, *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO mice retained some hypotensive response to AM. These results suggest that the hypotensive effect of AM is primarily mediated through the CLR/RAMP1 heterodimer, but that AM signaling via CLR/RAMP2 and CLR/RAMP3 also contributes to some hypotensive action. On the other hand, CGRP's hypotensive activity seems to be predominantly through the CLR/RAMP1 heterodimer. With this knowledge, therapeutic AM or CGRP peptides could be designed to cause less hypotension while maintaining canonical receptor-RAMP mediated signaling.

Keywords

Adrenomedullin; CGRP; RAMP; *Calcrl*; Hypotension; Blood pressure

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1. Introduction

Adrenomedullin (AM) is a circulating multi-functional peptide and a known vasodilator [1]. AM is a member of the calcitonin peptide family along with amylin and calcitonin gene-related peptide (CGRP), a peptide considered to be one of the most potent endogenous vasodilators [2]. Circulating levels of AM are increased during many forms of cardiovascular disease [3] including heart failure where AM plays a cardioprotective role.

Exogenous administration of AM has beneficial hemodynamic/renal effects, improved cardiac output, and overall survival rates [4–8]. AM imparts these cardioprotective actions via numerous biological functions including vasodilation, diuresis, and natriuresis which are further augmented by inhibition of the renin-angiotensin-aldosterone system [4–7]. In addition, exogenous AM is known to play a protective role in lung injury and sepsis, perhaps through its anti-inflammatory and anti-microbial properties [9,10]. Given the beneficial properties of AM peptide, it may serve as a candidate therapeutic agent. However, the vasodilatory effects of AM may cause undesirable side-effects or be contraindicated in some patients. Therefore, a more thorough understanding of the mechanisms underlying the vasodilatory action of AM may allow for the development of effective therapeutics.

AM and CGRP are ligands for the G-protein-coupled receptor known as calcitonin receptor-like receptor (CLR = protein name; *Calcrl* = gene name). In the endoplasmic reticulum, CLR associates with one of three receptor activity modifying proteins (RAMPs) that confer ligand specificity and binding affinity [11]. On their own, CLR and RAMPs rarely migrate to cell surface, but as a complex they translocate to the cell surface to interact with their respective ligands. Historically, the CLR/RAMP1 heterodimer is known as the CGRP receptor given its high ligand binding affinity for CGRP. CLR/RAMP2 and CLR/RAMP3 are known as the AM1 and AM2 receptors, respectively since AM is considered the primary ligand [12]. However, these receptor-RAMP heterodimers are not exclusively selective. AM can bind and activate the CGRP receptor and CGRP activates AM1 and AM2, though with lower potency than at the respective cognate receptors [13]

A 50% reduction in endogenous AM levels do not affect basal blood pressure in genetically heterozygous AM mice [14]. However, a bolus injection of AM causes dose-dependent hypotension [7,15]. The effects of exogenous AM on blood pressure have previously been examined using several genetic mouse models [16–18] and receptor inhibitors [19–21], but the relative contribution of each heterodimer to AM- and CGRP-induced hypotension has still not been fully explored or comparatively evaluated. Thus, the simultaneous examination of a comprehensive collection of genetic knockout mouse models for RAMPs and CLR could provide insight into the respective roles that CLR/RAMP1, CLR/RAMP2, and CLR/RAMP3 heterodimers play in the hypotensive responses to AM and CGRP.

In the current study, we examine the blood pressure and heart rate of mice under isoflurane anesthesia following intravenous injections of AM or CGRP. Gene disruption mouse models of *Calcrl*, *Ramp1*, *Ramp2*, and *Ramp3* were previously generated and are examined along with strain and age-matched wild type (WT) mice. Mice lacking *Calcrl* [22,23] and RAMP2 [18,23,24] are embryonic lethal, so these lines were examined as heterozygotes ($^{+/-}$).

2. Methods

2.1. Mice

All mice used in this study were between 8–16 weeks of age and are on the 129/S6-SvEv-TC1 background. The generation of *Ramp1*, *Ramp2*, *Ramp3* and *Calcrl* knockout mice were previously described [22,24,25]. All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

2.2. Blood pressure measurements

Mice were anesthetized with 2% isoflurane gas. The left carotid artery was exposed and a suture was placed around the distal end of the artery. Then a loosely tied suture was placed around the proximal end. An occluding ligature was placed on the proximal end of the artery to minimize bleeding during catheter insertion. A small incision was made in the isolated carotid artery near the distal end and a SciSense mouse blood pressure transducer was inserted. The proximal occlusion was then removed as the transducer was advanced into the artery retrograde to the ascending aorta and tied in placed. Measurements were recorded through a Scisense ADVantage PV control unit onto Labscribe 2 software (iWorx Systems, Inc.).

2.3. Peptides

AM and CGRP peptides (Phoenix Pharmaceuticals) were dissolved in saline solution (0.9% Sodium Chloride, Hospira). A volume of 0.05 ml was used for all vehicle control and experimental dosage intravenous injections, and an additional 0.02 ml of saline was injected to clear the catheter of peptide. In experiments where two identical doses of AM were injected 20 min apart (Fig. 2), 12 nmol/kg of AM peptide was used per dose. In experiments where sequentially increasing doses of AM or CGRP were injected 5 min apart, 0.12 nmol/kg of peptide was used for the first dose, 1.2 nmol/kg for the second dose, 12 nmol/kg for the third dose, and 120 nmol/kg for the fourth and final dose.

2.4. Data and statistical analysis

Basal blood pressure was defined as the mean arterial pressure (MAP) during the 2 min prior to the first injection. Using Labscribe 2 software, MAP was calculated by applying a smoothing function to blood pressure charts, averaging each data point (1000/sec) by all neighboring data points within 1.5 s. Changes in blood pressure following injection were determined by identifying the lowest MAP measurement after injection and calculating the percent change from baseline blood pressure. Percent change was used, rather than reporting absolute blood pressure, because significant differences in basal blood pressure exist between mouse lines and sexes. Statistical analyses were performed using the Student's *t*-test. Differences with $P < 0.05$ were deemed statistically significant.

In experiments where mice received two 12 nmol/kg doses of AM 20 min apart (Fig. 2) each genotype was $n \geq 8$ when both sexes are combined and $n \geq 4$ for each sex alone. Specifically, WT mice were n = 12 (7 males, 5 females), *Ramp1*$^{-/-}$ mice were n = 8 (4 males, 4 females), *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO mice were n = 11 (5 males, 6 females), *Ramp3*$^{-/-}$ mice were n = 14 (8 males, 6 females), *Ramp2*$^{+/-}$ mice were n = 8 (4 males, 4 females), and *Calcrl*$^{+/-}$ mice

were n = 8 (4 males, 4 females). In experiments where four sequentially increasing doses (0.12 nmol/kg to 120 nmol/kg) of peptide were injected 5 min apart, mice receiving AM were n = 5 (2 males, 3 females) per genotype, and mice receiving CGRP were n = 4 (2 males, 2 females) per genotype. Baseline blood pressure and heart rate were measured under anesthesia, prior to dosing, and calculations in Table 1 were made from all the mice used in the experiments just previously described.

3. Results

3.1. Basal blood pressure and heart rate

The basal blood pressures of anesthetized male and female mice were compared to wildtype (WT) mice of the same sex. *Ramp1*$^{-/-}$, *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO, and *Calcrl*$^{+/-}$ males were each found to have significantly higher basal blood pressure than their sex-matched WT counterparts (Table 1). We also analyzed for sexual dimorphism within each genotype. WT control males showed a modestly elevated basal blood pressure than WT females, but this difference was not statistically significant. In contrast, *Ramp1*$^{-/-}$, *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$, *Ramp3*$^{-/-}$, and *Calcrl*$^{+/-}$ males all showed significantly elevated blood pressures compared to female mice of the same genotype. We also examined baseline heart rate, and found no significant differences to sex-matched WT mice; however we did find a reduction in *Calcrl*$^{+/-}$ female heart rate compared to *Calcrl*$^{+/-}$ males (Table 1).

3.2. Effect of exogenous AM and CGRP on blood pressure

Using WT mice, 12 nmol/kg of AM was empirically determined to be an appropriate dose to elicit a robust and measureable decline in blood pressure that was large enough to observe any attenuation that may occur in the genetic knockout models. To ensure proper insertion of the catheter prior to administering AM, each mouse was initially injected with saline solution (vehicle) and no measurable response was observed following vehicle injection (data not shown). Following intravenous injection of 12 nmol/kg of AM, WT mouse blood pressure declined by 13.0 ± 1.2% (n = 14) and remained depressed with a gradual recovery in the following minutes. An identical dose, administered 20 min later to the same animal, caused an additional drop of 11.3 ± 0.8% (n = 12) (Figs. 1 and 2A, B). This effect was attenuated in *Ramp1*$^{-/-}$ mice, and to a lesser extent, in *Ramp3*$^{-/-}$ and *Calcrl*$^{+/-}$ mice. The first injection of AM in *Ramp1*$^{-/-}$ mice reduced blood pressure by about 52% of what was observed in WT animals (6.2 ± 1.0% n = 8, $p < 0.0001$). However, the second AM dose resulted in only a slight drop in mean pressure (1.7 ± 0.8% n = 7, $p < 0.0001$) with 4 out of 7 mice showing no measurable change in blood pressure. In *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO mice, similar results were observed for the first (6.1 ± 1.6% n = 11, $p < 0.0001$) and second (0.8 ± 0.4% n = 11, $p < 0.0001$) AM injection, with 7 out of 11 mice showing no change in blood pressure on the second dose. Examination of heart rate following AM injections did not show any significant difference between genotypes (data not shown).

Results for *Ramp1*$^{-/-}$ and *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO mice were similar when separately examining males and females (Fig. 2C–F). Compared to WT, *Ramp3*$^{-/-}$ mice showed a significantly diminished decline in blood pressure following the first injection (9.3 ± 1.4% n = 14, $p < 0.05$), but the second injection (10.1 ± 0.6% n = 14, ns) showed no significance

and was similar to the first. Subsequent analysis by sex of *Ramp3*$^{-/-}$ mice showed no difference in AM responsiveness to their WT counterparts. *Ramp2*$^{+/-}$ mice behaved similarly to WT for both the first (12.3 ± 1.2% n = 8, ns) and second (12.6 ± 2.0% n = 7, ns) AM injections. The decline in blood pressure was diminished by 29% in *Calcrl*$^{+/-}$ mice with the first AM dose (9.2 ± 1.0% n = 8, $p < 0.05$) and by 28% after the second AM dose (8.1 ± 0.7% n = 8, $p < 0.05$). *Calcrl*$^{+/-}$ males, but not *Calcrl*$^{+/-}$ females, showed a significantly attenuated response to exogenous AM compared to WT controls. However, when directly compared, there was statistically no difference between *Calcrl*$^{+/-}$ males and females.

To determine how AM-induced hypotension contrasts with CGRP-induced hypotension, sequentially increasing doses ranging from 0.12 nmol/kg to 120 nmol/kg of AM (Fig. 3A) or CGRP (Fig. 3B) were administered 5 min apart. This experiment revealed that AM is between 10 and 100 fold less potent that CGRP in eliciting a hypotensive response in WT mice. However, in *Ramp1*$^{-/-}$ mice the CGRP response is effectively eliminated ($p = 0.003$, paired *T*-test of *Ramp1*$^{-/-}$ to WT at 12 nmol/kg CGRP); these mice retain a response to AM, though the magnitude of response is reduced by ~50% compared to WT ($p = 0.002$, paired *T*-test of *Ramp1*$^{-/-}$ to WT at 12 nmol/kg AM) (Fig. 3A, B). Knockout of *Ramp3*, either alone or in combination with *Ramp1*, does not appear to impart any significant effect on the hypotensive response to either AM ($p = 0.390$, paired *T*-test of *Ramp3*$^{-/-}$ to WT at 12 nmol/kg) or CGRP ($p = 0.988$, paired *T*-test of *Ramp3*$^{-/-}$ to WT at 12 nmol/kg). Additionally, the highest dose of 120 nmol/kg CGRP was relatively ineffective in WT, *Ramp3*$^{-/-}$, *Ramp2*$^{+/-}$, and *Calcrl*$^{+/-}$ mice perhaps due to receptor internalization and desensitization. To determine if the desensitization effect caused by CGRP was able to alter the hypotensive response to AM, we repeated the experiment, but gave 120 nmol/kg AM instead of CGRP at the last dose (Fig. 3C). AM still gave a hypotensive response (Fig. 3C), but at a similar level observed in AM-only treated *Ramp1*$^{-/-}$ mice (Fig. 3A).

4. Discussion

4.1. Sexual dimorphisms in basal blood pressure

Although not statistically significant in the WT group, we find a consistent trend of higher blood pressure in males over females across all genotypes (Table 1). With *Ramp2*$^{+/-}$ being the only exception, this sexually dimorphic effect is observed in all mice tested, and appears to be imparted primarily by an increase in male blood pressure, rather than a significant reduction in female blood pressure. This suggests that males may be more susceptible to disruptions in CLR-mediated signaling which may contribute to the development or exacerbation of a disease state. In fact, in a chronic hypertension model [26], male mice with a 50% reduction in endogenous AM have increased cardiac hypertrophy and renal damage which were not observed in female mice [14]. Furthermore, evidence by our group using the same chronic hypertension model showed a similar sex dependent effect on cardiac hypertrophy in *Ramp3*$^{-/-}$ mice [27].

Previous studies on CGRP KO mice have provided contrasting evidence on the effect of endogenous CGRP in baseline blood pressure. CGRP KO mice generated by Lu et al. show no change in baseline blood pressure [28], whereas CGRP KO mice on a hybrid 129/Sv x C57BL/6 background exhibit hypertension [29]. Indeed, sex differences in blood pressure

are highly strain dependent. For example, C57BL/6J female mice have higher blood pressure than their male counterparts, whereas 129S1/SvImJ, a strain more closely related to the one used in this study (129S6/SvEv-Tac1), have the opposite relationship [30]. It's unclear if disrupted signaling via CLR would show similar or contrasting effects on blood pressure in other genetic strains, but our observations suggest that within the genetic diversity of the human population some individuals may have altered blood pressure based on differences in CLR-mediated signaling. Previous findings in humans showing that infusion of CGRP antagonists have no effect on resting blood pressure seem to suggest otherwise [31], but a short agonist treatment may not be analogous to the complete and consistent effect of genetically altered CLR-mediated signaling.

4.2. Effect of exogenous AM and CGRP on blood pressure

In the present study, *Ramp1*$^{-/-}$, *Ramp2*$^{+/-}$, *Ramp3*$^{-/-}$, *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO and *Calcrl*$^{+/-}$ mice were systematically and comparatively examined to determine the effects of exogenous AM and CGRP on blood pressure and heart rate. *Ramp2*$^{+/-}$ and *Calcrl*$^{+/-}$ mice are used because of the embryonic lethality of *Ramp2*$^{-/-}$ and *Calcrl*$^{-/-}$ mice. *Calcrl*$^{+/-}$ mice showed a modest, but significant attenuation in AM-induced hypotension. This is an expected outcome, considering that the biological effects of AM are mediated via signaling through heterodimers with the common CLR receptor. What has not been clear is to what extent each of the RAMPs is involved in modulating the effect of AM on blood pressure when dimerized with CLR. We demonstrate here that *Ramp1*$^{-/-}$ mice showed a significantly diminished blood pressure response following an intravenous injection of AM. This has previously been observed in sodium pentobarbital anesthetized *Ramp1*$^{-/-}$ mice and fits with previous observations that the hypotensive effect of exogenous AM is attenuated in the presence of the CLR/RAMP1 antagonist peptide, CGRP(8–37) [16,19]. However, it should be noted that despite AM and CGRP both causing a hypotensive response via CLR/RAMP1, it has been shown in small human thymic arteries [32] and sheep coronary artery [33] that AM causes vasodilation via a nitric oxide-guanylyl cyclase mechanism, whereas CGRP was found to signal through a cAMP-dependent mechanism [32]. Additionally, results from endothelial denudation experiments suggest that AM primarily induces vasodilation through the endothelium, whereas CGRP does not [32]. So, common signaling through CLR/RAMP1 does not preclude AM and CGRP from signaling via divergent downstream pathways that may be cell type dependent.

The blood pressure response after the second dose of AM in *Ramp1*$^{-/-}$ mice is significantly more diminished than the response elicited by the first injection (Fig. 2A, C, and E). This is likely due to desensitization and internalization of occupied receptors during the initial dose [34]. Compared to WT, *Ramp1*$^{-/-}$ mice exhibited over a 50% reduction of hypotension caused by the initial dose of AM and a greater than 80% reduction caused by the second dose of AM. This suggests that the AM-induced hypotension is primarily mediated through the CLR/RAMP1 heterodimer, which has historically been considered the CGRP receptor. However, even at an affinity of 1–2 orders of magnitude lower than CGRP, the affinity of AM for CLR/RAMP1 still has physiological effects [35,36]. With the CLR/RAMP1 heterodimer established as a primary mediator of the hypotensive response, it stands to

reason that AM causes a hypotensive response similar to CGRP at a dosage of 1–2 orders of magnitude higher, given their relative affinities.

Since a modest reduction of blood pressure still occurs following AM treatment in *Ramp1*$^{-/-}$ mice, it would be expected that the remainder of signaling would be mediated through the CLR/RAMP2 and CLR/RAMP3 heterodimers. Direct examination of RAMP2 via genetic deletion is precluded by embryonic lethality of the full knockout mouse, and *Ramp2*$^{+/-}$ mice do not show an attenuated response to AM, perhaps due to a sufficient amount of RAMP2 remaining to generate a normal blood pressure response. So to further elucidate the relative contributions of these heterodimers to the AM-induced hypotension, *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO mice were examined, leaving RAMP2 as the only RAMP expressed. This analysis did not reveal any significant difference in AM-induced hypotension between *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO and *Ramp1*$^{-/-}$ mice, and *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO mice still exhibited a hypotensive response. Since RAMP2 is the only RAMP expressed in the *Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$ dKO, this suggests that the hypotensive response of AM is mediated through both CLR/RAMP1 and CLR/RAMP2, whereas the effect of CGRP is almost exclusively mediated by CLR/RAMP1. This is further supported by our finding that desensitization to CGRP after sequentially increasing doses did not fully attenuate the hypotensive response caused by AM (Fig. 3C), and the observation that *Calcrl*$^{+/-}$ mice do not seem any more desensitized than WT mice to CGRP after treatment (Fig. 3B) suggests that it is likely CLR-RAMP1 depletion, but not CLR-RAMP2/3 depletion, that is the cause for this effect. We did see a significant attenuation of hypotension to AM after the first injection in *Ramp3*$^{-/-}$ mice, but this was only observed after pooling data from both sexes and was not recapitulated by the second injection.

Interpretation of the results from mice deficient in RAMP2 and RAMP3 is complicated by the fact that loss of a high affinity binding receptor increases available ligand for CLR/RAMP1 which would presumably enhance the hypotension in an initial AM dose. Additionally, with the removal of competition for available CLR in the endoplasmic reticulum there may be increased availability of CLR for RAMP1 heterodimerization which could lead to increased surface CLR/RAMP1. A third layer of influence could reside in compensatory alterations in relative gene expression levels of non-targeted RAMPs and CLR in the knockout models. For example, we previously showed that *Ramp3*$^{-/-}$ mice have increased *Calcrl* mRNA expression [27], but it is not clear if this would result in more CLR/RAMP1 on the cell surface.

Direct examination of the hypotensive effects of AM in the absence of the CLR/RAMP2 heterodimer is precluded by the embryonic lethality of *Ramp2*$^{-/-}$ mice. In this study, *Ramp2*$^{+/-}$mice showed no significant differences compared to WT mice. However, mice overexpressing RAMP2 in smooth muscle cells have an enhanced hypotensive effect to exogenous AM following intravenous injection [17], which supports a clear role for the CLR/RAMP2 heterodimer in the hypotensive response to AM. More recently, we have developed a genetic mouse model in which the expression of *Ramp2* is deleted in all cells, except cells expressing the endothelial VE-cadherin promoter [37], thus imparting an ability of some of these animals to survive embryonic lethality. These mice show marked systemic hypotension, once again supporting a role for the CLR/RAMP2 heterodimer in regulating

basal blood pressure. Unfortunately, because *Ramp2* and *Ramp3* reside very closely to each other on mouse chromosome 11, the generation of a RAMP2/3 dKO or RAMP triple-KO animal remains challenging. As such, we cannot fully eliminate the possibility that some of the AM-induced hypotension may be mediated via a non-canonical pathway or mechanism. Nevertheless, these results along with previously published observations suggest that either or both CLR/RAMP2 and CLR/RAMP3 heterodimers play an important role in mediating the hypotensive response to AM.

4.3. Therapeutic implications as evident from disease models

Several lines of evidence suggest that both AM and CGRP have cardioprotective effects that may be therapeutically valuable. In a DOCA-salt hypertension model, CGRP/calcitonin knockout mice are more vulnerable to hypertension-induced heart and kidney damage [38] which may be attributed to its role as a suppressor of inflammatory mediators [16]. In a transverse aortic constriction model of heart disease, CGRP knockout mice had reduced survivability and a worsened cardiac phenotype [39]. Inversely, infusion of CGRP has been shown to improve circulation in patients with heart disease [40,41]. When $Adm^{+/-}$ mice were given an AngII/high-salt treatment, they had worsened perivascular fibrosis and intimal hyperplasia in the coronary arteries compared to WT mice, possibly via inhibition of oxidative stress [42]. In a rat model of heart failure, acute administration of AM increased cardiac output and reduced right ventricular systolic pressure only in failing hearts. Subsequently, in patients with heart failure, intravascularly administered AM improved cardiac index and reduced pulmonary arterial pressure only in individuals experiencing heart failure. Additionally, administration of AM in the $ApoE^{-/-}$ mouse model of spontaneous atherosclerosis reduced the appearance of atherosclerotic lesions [43].

In addition to their cardioprotective effects, these peptides have a wide range of other functions including in the reproductive system [44], the lymphatic vasculature [23], and the nervous system [45], and so their therapeutic potential must be evaluated in the context of broader systemic functions [46]. Given the wide range of actions of these peptides, continuing to study how AM and CGRP signal to produce these actions may help in the development of improved therapeutic peptides that could be tailored to the specific disease indication.

5. Conclusions

This work highlights the importance of CLR-RAMP signaling in AM-induced hypotension and establishes CLR/RAMP1 as the primary, but not sole, mediator of the effect. These results further suggest CLR/RAMP2 and CLR/RAMP3 contribute to the remaining hypotensive effect, while CGRP induced hypotension signaling remains specific for the CLR/RAMP1 heterodimer. While the sex of an individual has an established contribution to differences in blood pressure, this works shows no obvious sexual dimorphism in the response to infusion of AM or CGRP. We hope that these contributions provide an improved understanding of AM/CGRP peptide effects on blood pressure regulation, with important implications for future therapeutic strategies.

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Abbreviations

AM	adrenomedullin
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor protein
Calcrl	calcitonin receptor-like receptor gene
MAP	mean arterial pressure
RAMP	receptor activity modifying protein
dKO	double knockout

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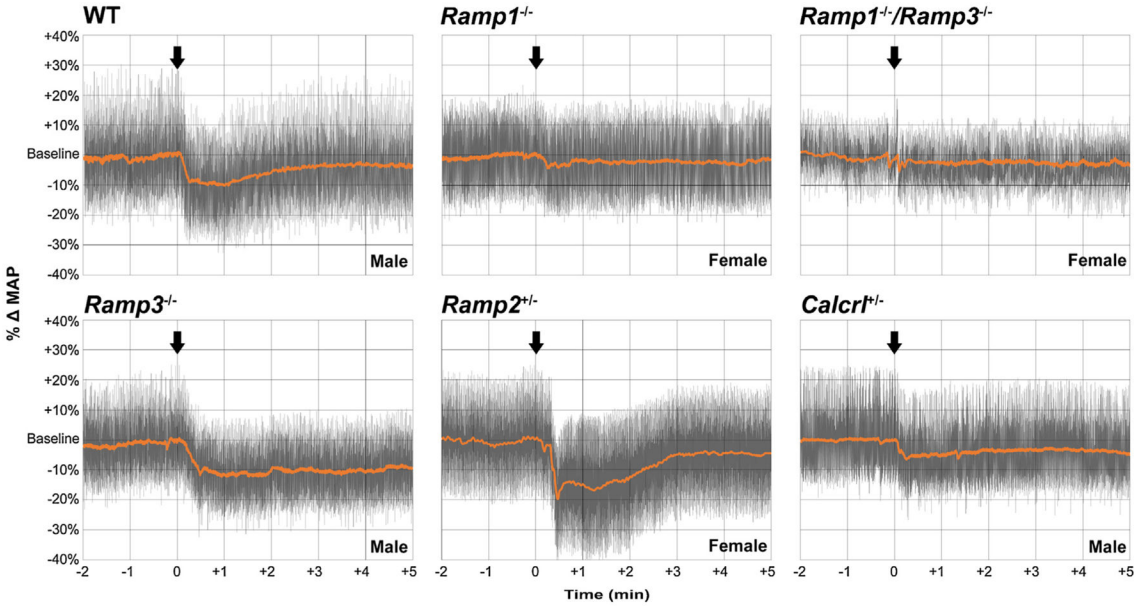

Fig. 1.

Representative blood pressure charts from a single mouse receiving a 12 nmol/kg dose of AM converted to a percentage of baseline pressure (black), and the smooth line calculated from the chart (orange). Time 0 marks the time of AM injection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

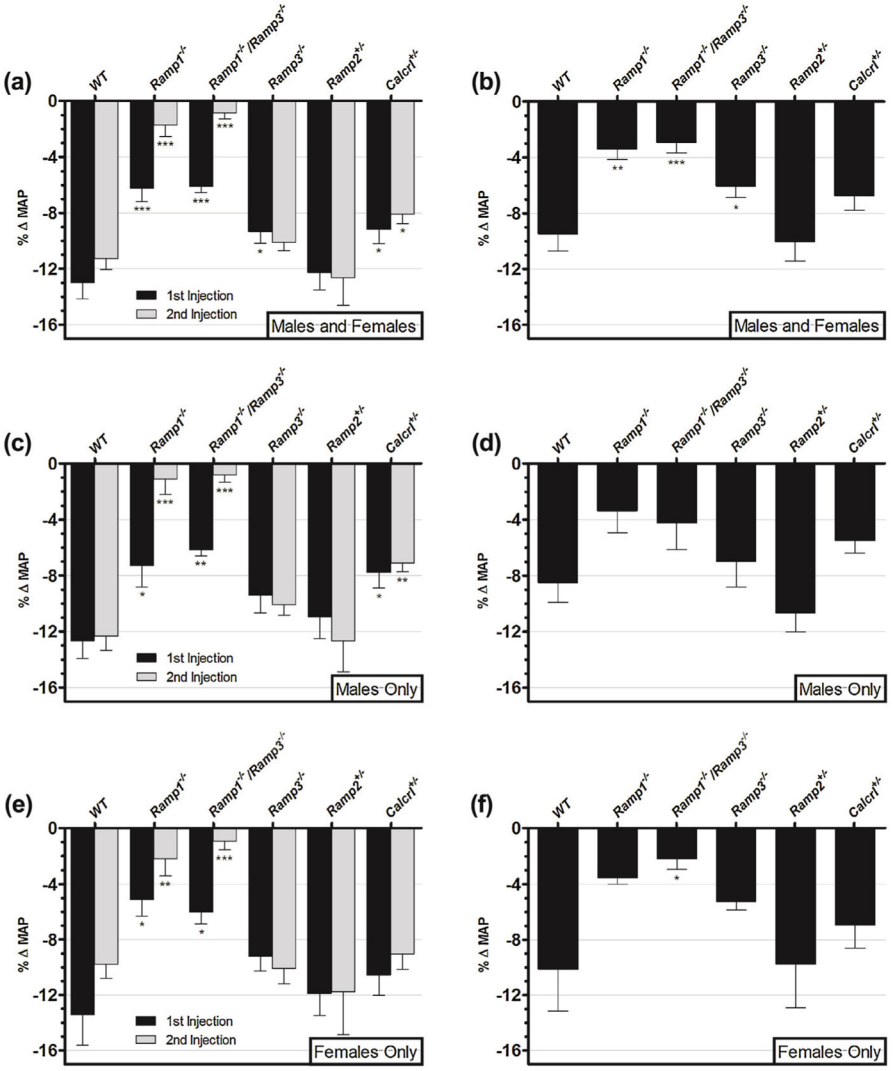

Fig. 2.

Change in blood pressure following 12 nmol/kg intravenous injections of AM. Injections were spaced 20 min apart. (a,c,e) Maximum acute change in blood pressure after two injections, 20 min apart, for (a) all mice, (c) males, and (e) females. (b,d,f) Mean blood pressure of the first 3 min following the first injection for (b) all mice, (d) males, and (f) females. For all analyses, $n \geq 8$ for all genotypes and $n \geq 4$ per sex of each genotype. Significance to WT after the same injection (*) is shown. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

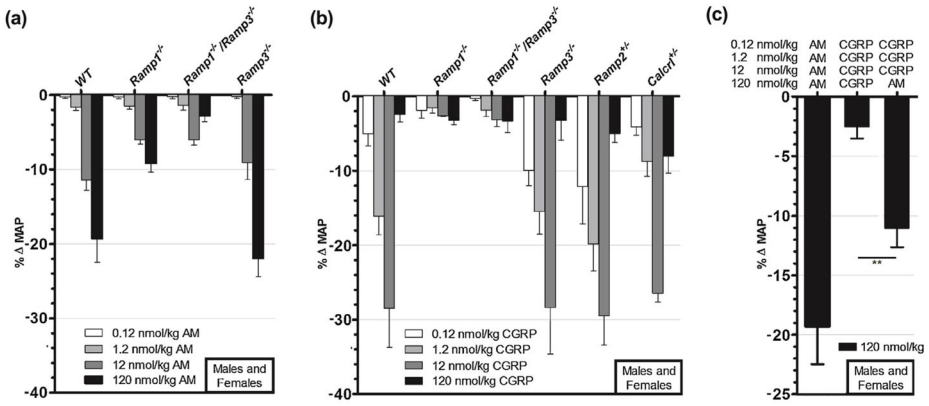

Fig. 3.

The change in blood pressure following sequentially increasing doses of (a) AM and (b) CGRP from 0.12 nmol/kg to 120 nmol/kg body weight. (c) WT mice were given sequentially increasing doses of CGRP up to 12 nmol/kg, same as panel b, but the 4th and final dose given was 120 nmol/kg of AM (n = 4) instead of CGRP (n = 4). All doses were administered 5 min apart. Each genotype receiving AM were n = 5 mice composed of 3 females and 2 males. Each genotype receiving CGRP were n = 4 mice composed of 2 females and 2 males. The effect of AM and CGRP on blood pressure across genotypes and dosages were each significant by two-way analysis of variance with P-values < 0.0001. ** P < 0.01, Student's *t*-test.

Table 1

Baseline blood pressure (mmHg) and heart rate (bmp, beats per minute) of isoflurane anesthetized mice.

		WT	*Ramp1*$^{-/-}$	*Ramp1*$^{-/-}$/*Ramp3*$^{-/-}$	*Ramp3*$^{-/-}$	*Ramp2*$^{+/-}$	*Calcrl*$^{+/-}$
Male	BP (mmHg)	90.15 ± 2.91	101.5 ± 5.84†,*	98.64 ± 2.02†††,*	96.03 ± 3.04†††	95.68 ± 3.86	101.6 ± 4.10††,*
	HR (bpm)	450.9 ± 8.7	440.5 ± 17.2	457.6 ± 10.0	436.9 ± 10.7	415.5 ± 18.5	450.0 ± 11.7††
Female	BP (mmHg)	81.18 ± 2.66	85.64 ± 1.67	79.06 ± 3.53	77.45 ± 1.86	86.65 ± 2.81	84.12 ± 1.90
	HR (bpm)	434.0 ± 15.8	448.8 ± 16.2	424.7 ± 20.2	421.6 ± 13.5	389.0 ± 19.3	388.7 ± 10.4

For all analyses, n ≥ 8 for all genotypes and n ≥ 4 per sex of each genotype. Significance to WT of the same sex (*) and significance to females of the same genotype (†) are shown.

*P < 0.05.

†P < 0.05.

††P < 0.01.

†††P < 0.001.