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Receptor Activity-modifying Proteins 2 and 3 Have Distinct Physiological Functions from Embryogenesis to Old Age*

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RAMPs (receptor activity modifying proteins) impart remarkable effects on G protein-coupled receptor (GPCR) signaling. First identified through an interaction with the calcitonin receptor-like receptor (CLR), these single transmembrane proteins are now known to modulate the *in vitro* ligand binding affinity, trafficking, and second messenger pathways of numerous GPCRs. Consequently, the receptor-RAMP interface represents an attractive pharmacological target for the treatment of disease. Although the three known mammalian RAMPs differ in their sequences and tissue expression, results from *in vitro* biochemical and pharmacological studies suggest that they have overlapping effects on the GPCRs with which they interact. Therefore, to determine whether RAMP2 and RAMP3 have distinct functions *in vivo*, we generated mice with targeted deletions of either the *RAMP2* or *RAMP3* gene. Strikingly, we found that, although RAMP2 is required for survival, mice that lack RAMP3 appear normal until old age, at which point they have decreased weight. In addition, mice with reduced expression of *RAMP2* (but not *RAMP3*) display remarkable subfertility. Thus, each gene has functions *in vivo* that cannot be accomplished by the other. Because RAMP2, RAMP3, and CLR transduce the signaling of the two potent vasodilators adrenomedullin and calcitonin gene-related peptide, we tested the effects of our genetic modifications on blood pressure, and no effects were detected. Nevertheless, our studies reveal that RAMP2 and RAMP3 have distinct physiological functions throughout embryogenesis, adulthood, and old age, and the mice we have generated provide novel genetic tools to further explore the utility of the receptor-RAMP interface as a pharmacological target.

The identification of receptor activity modifying proteins (RAMPs)[2] 1–3 has revolutionized our current understanding of the mechanism through which class II G protein-coupled receptors (GPCRs) bind to their peptide ligands. First identified in association with the calcitonin receptor-like receptor (CLR; formerly called CRLR), either of these three single pass transmembrane proteins can bind to a GPCR, chaperone it to the plasma membrane, and alter the ligand binding affinity of the receptor (1). For example, a CLR·RAMP1 complex preferentially and specifically binds to calcitonin gene-related peptide (CGRP), whereas a CLR·RAMP2 or CLR·RAMP3 complex will preferentially bind to adrenomedullin (AM), another peptide vasodilator. Thus, the different spatial and temporal expression patterns of RAMP1, RAMP2, and RAMP3 determine how a cell or tissue will sense and respond to either extracellular CGRP or AM.

Biochemical studies using heterologous overexpression of RAMPs in cultured cells have demonstrated that this general mechanism also applies to several other GPCRs of the class II family, including calcitonin receptor, parathyroid receptors 1 and 2, vasointestinal peptide/pituitary adenylate cyclase-activating peptide 1 (VIP/VPAC1) receptor, and glucagon receptor (2, 3). More recently, Bouschet *et al.* (4) have also demonstrated that RAMP1 or RAMP3 can functionally target a class III receptor, the calcium sensing receptor, to the plasma membrane. Therefore, it is likely that RAMP proteins have evolved to impart a highly controllable mechanism for modulating GPCR signaling that may be broadly applicable to many GPCRs (5). As a consequence, the pharmacological and biochemical study of the RAMP-receptor interaction has been geared toward identifying compounds that exploit this interface as a potential drug target for the specific modulation of GPCR signaling for the treatment of human disease (6). One such compound, BIBN4096BS, which is currently in clinical trials for the treatment of migraine, acts as a selective CGRP antagonist by interfering with the hCLR·hRAMP1 interaction (7, 8). Yet, the developmental and physiological consequences of genetically altering RAMP function or expression in the whole animal have not been addressed experimentally.

Receptor-associated RAMPs have been linked with receptor glycosylation, receptor trafficking, ligand binding, and alteration of second messenger signaling (recently reviewed in Refs. 9 and 10). However, these ascribed cellular functions are not consistently conserved among the different receptor·RAMP complexes and are highly affected by the choice of cell type and species of RAMPs studied (9). Moreover, pharmacological studies with the most well characterized receptor·RAMP pairs, CLR·RAMP1–3 and calcitonin·RAMP1–3, demonstrate varying degrees of overlap in the absolute ligand binding affinity imparted by the different RAMPs. For example,

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[2] The abbreviations used are: RAMP, receptor activity modifying protein; GPCR, G protein-coupled receptor; CLR, calcitonin receptor-like receptor; CGRP, calcitonin gene-related peptide; AM, adrenomedullin; RT, reverse transcription.

CLR·RAMP1–3 complexes can all bind AM or CGRP but with different affinities (11–13), and calcitonin·RAMP1–3 complexes can form amylin receptors with highly variable affinities (3, 14) or CGRP receptors (15, 16), depending on the cell type studied. Thus, it remains unclear to what degree the three mammalian RAMPs have overlapping functions or whether they can functionally compensate for each other *in vivo*. To address these questions, we used gene targeting to generate mice with targeted deletions of either the *RAMP2* or *RAMP3* genes and have determined the effects of their complete absence (in homozygous null mice) and of their reduced expression (in heterozygous mice).

EXPERIMENTAL PROCEDURES

Generation of Mice with Targeted Deletion of the RAMP2 or RAMP3 Gene—To generate the targeting vectors, a 129S6/SvEv genomic library was screened for phage clones containing the 5′ portions of the *RAMP2* and *RAMP3* genes using DNA fragments isolated from *hRAMP2* or *hRAMP3* expression plasmids (kindly provided by Dr. Steven Foord, GlaxoSmithKline). Using convenient restriction sites within the genomic clones, 5′ and 3′ regions of homology were subcloned into the multiple cloning site of a gene-targeting vector that contained a phosphoglycerate kinase-neomycin cassette and an a herpes simplex virus-thymidine kinase cassette. The final targeting vectors were linearized with NotI before electroporation into embryonic stem cells.

Standard gene targeting methods were utilized to generate embryonic stem cells and mice with a targeted deletion of the *RAMP2* gene or a targeted deletion of the *RAMP3* gene (17). Briefly, 129S6/SvEv-TC-1 embryonic stem cells were electroporated with the linearized targeting vectors shown in Figs. 1*A* and 2*A*, respectively. After applying positive (G418) and negative (gancyclovir) selection, positive embryonic stem cell clones were identified by Southern blot and/or PCR. The frequency of homologous recombination in the surviving G418/gancyclovir-resistant colonies was 5% for *RAMP2* and 1.5% for *RAMP3*. Male chimeric mice that transmitted the targeted allele were bred to 129S6/SvEv females to establish isogenic lines.

For PCR-based genotyping of the *RAMP2*-targeted locus, we used the following three primers: primer 1, 5′-CTGAACTGAACAGCAGGGCCA-3′; primer 2, 5′ CGGCTACTTCCCACTTAATGCTG-3′; and primer 3, 5′GCTTCCTCTTGCAAAACCACA-3′. Primers 1 and 3 amplify a 1.2-kb-targeted band, whereas primers 1 and 2 amplify a 1.6-kb wild type band.

For PCR-based genotyping of the *RAMP3*-targeted locus, we used the following four primers: primer 1, 5′-GCCCATGATGTTGGTCTCCA-3′; primer 2, 5′ GGTCATTAGGAGCCACGTGT-3′; primer 3, 5′GCTTCCTCTTGCAAAACCACA-3′; and primer 4, 5′GGGCTAAAGAAGCCACAGCT-3′. Primers 1 and 3 amplify a 2.0-kb-targeted band, whereas primers 2 and 4 amplify a 1.4-kb wild type band.

Gene Expression Analysis—*RAMP2*, *RAMP3*, and *calcrl* gene expression was analyzed by quantitative reverse transcription (RT)-PCR with the Mx3000P Q-PCR machine from Stratagene. Primers for *RAMP2* amplification were 5′-CAGAATCAATCTCATCCCACTGAC-3′ and 5′-GTCCATGCAACTCTTGTACTCATACC-3′. The probe sequence for *RAMP2* detection was 5′-FAM-ATGGAAGACTACGAAACACATGTCCTACCTTG-TAMRA-3′. Primers for *RAMP3* amplification were 5′-GGTCATTAGGAGCCACGTGT-3′ and 5′-GGGCTAAACAAGCCACAGCT-3′. The probe sequence for *RAMP3* detection was 5′-FAM-CACGATTCTGTGTCCAGTGTGGGCTG-TAMRA-3′. Primer and probe sequences for detection of *calcrl* gene expression were previously described (18). β-Actin served as an internal control for all reactions. The primers used for *β-actin* amplification were 5′-CTGCCTGACGGCCAAGTC-3′ and 5′-CAAGAAGGAAGGCTGGAAAAGA-3′. The probe sequence for *β-actin* detection was 5′-TET-CACTATTGGCAACGAGCGGTTCCG-TAMRA-3′. RNA was isolated from adult tissues or embryonic day 13.5 embryos with TRIzol reagent (Invitrogen) and subsequently DNase treated and purified with an RNeasy Mini kit (Qiagen). 200 ng of total RNA was used in each reaction. The ΔΔCt method (19) was used to determine the relative levels of gene expression and shown as a percentage of wild type. All assays were repeated three times, each with duplicates.

Measurement of Basal Blood Pressure, Heart Rate, and Urine Chemistry—Blood pressures and heart rates were measured on unanesthetized mice by a computerized tail cuff system (20). Urine and protein creatinine were measured at the University of North Carolina-Chapel Hill Animal Clinical Chemistry Core Facility.

Experimental Animals—Unless otherwise noted, experimental animals were 4–8 months old and maintained on an isogenic 129S6/SvEv-TC-1 background. Control animals for all experiments consisted of wild type age- and gender-matched littermates. All experiments were approved by the Institutional Animal Care and Use Committee of The University of North Carolina-Chapel Hill.

Statistics—Statistical analyses for multiple comparisons were performed with one way analysis of variance by JMP Software, SAS Institute. Error bars represent S.E. of the means. Differences were considered significant with a p value of <0.05.

RESULTS

Generation of Heterozygous Mice with Targeted Disruption of the RAMP2 Gene—Mice in which exons 1 and 2 of the *RAMP2* gene were deleted by homologous recombination were generated using the targeting strategy shown in Fig. 1*A*. The disrupted allele, which lacks the *RAMP2* promoter, 5′-untranslated region translation start site, and exons 1 and 2, was confirmed by genomic PCR (Fig. 1*B*). To confirm that the gene targeting effectively disrupted transcription of the *RAMP2* gene, quantitative reverse transcription-PCR for *RAMP2* RNA was performed on total RNA isolated from whole embryos. As expected and shown in Fig. 1*C*, $RAMP2^{+/-}$ embryos expressed approximately half the wild type *RAMP2* RNA levels (58%, $p < 0.001$ *versus* wild type), whereas $RAMP2^{-/-}$ embryos had no detectable levels of *RAMP2* RNA, thus confirming correct gene targeting of the *RAMP2* gene (Fig. 1*C*). To determine whether genetic reduction of RAMP2 caused a homeostatic compensation in the expression of the *RAMP3* or *calcrl* genes, we measured the expression of these genes in the heart and kidneys of adult $RAMP2^{+/-}$ animals and in total RNA isolated from mid-gestation $RAMP2^{-/-}$ embryos. As shown in Fig. 1*D*,

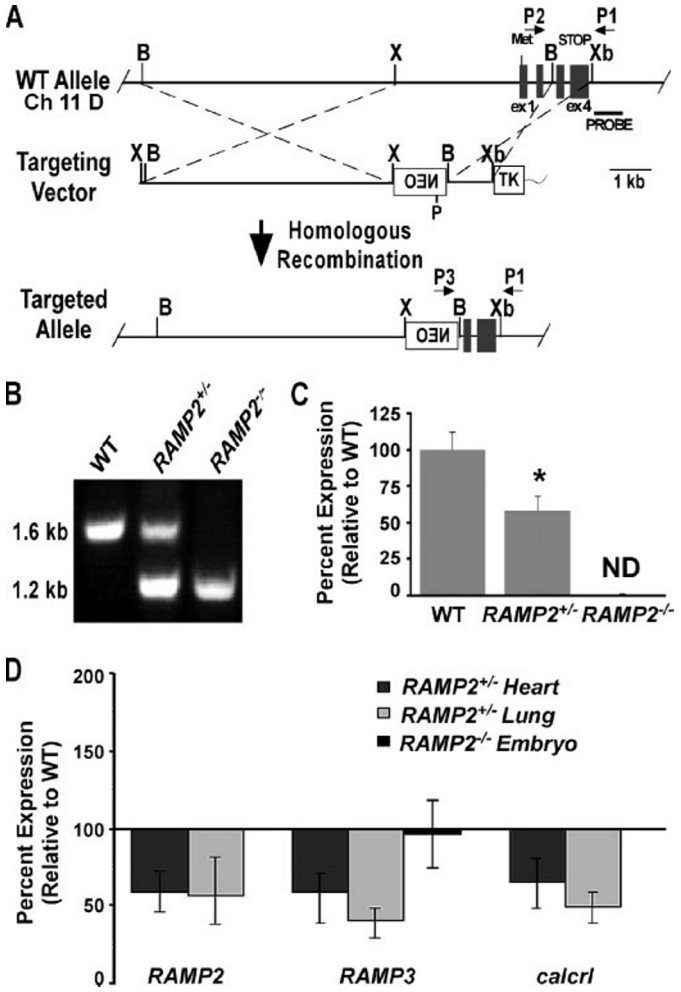

FIGURE 1. **Gene targeting of *RAMP2*.** *A*, schematic representation of the strategy used to disrupt the *RAMP2* gene in mice. The promoter region and exons 1 and 2 of *RAMP2*, including the initiator methionine were deleted by homologous recombination. *B*, BamHI X, Xho, Xb, and Xba and P1, P2, and P3 primers used for screening and genotyping. *B*, genomic PCR for detection of the wild type and targeted alleles using total embryonic DNA extracts and the primers depicted in Fig. 1*A*. *C*, quantitative RT-PCR for detection of *RAMP2* mRNA transcripts using total embryonic RNA extracts and the primers and probes described under "Experimental Procedures." *ND*, not detected. *D*, quantitative RT-PCR for detection of *RAMP2*, *RAMP3*, and *calcrl* mRNA transcripts using total RNA extracted from the hearts and lungs of $RAMP2^{+/-}$ adult mice and $RAMP2^{-/-}$ embryos.

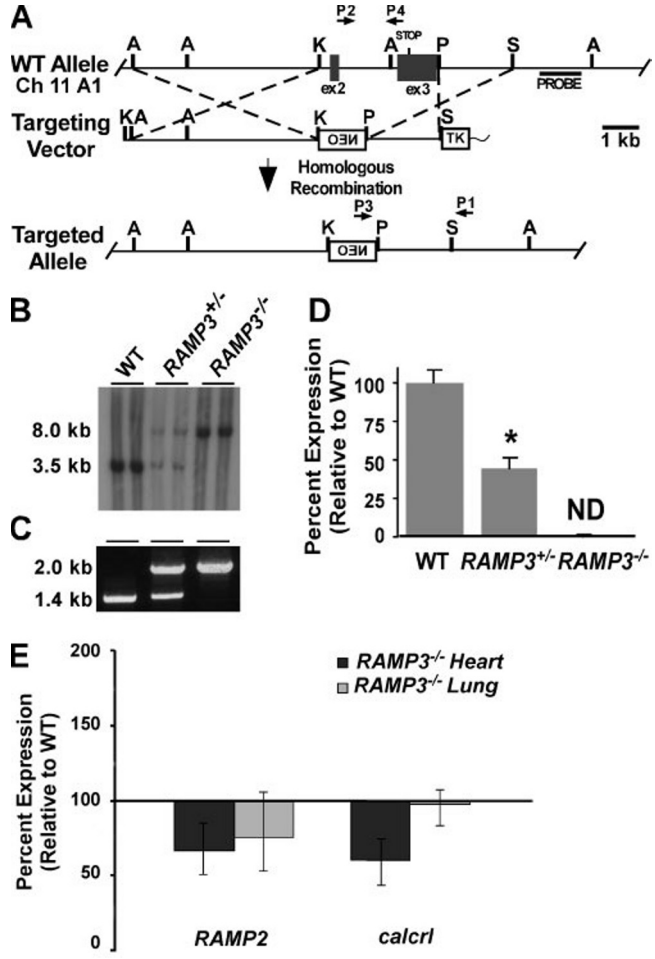

FIGURE 2. **Gene targeting of *RAMP3*.** *A*, schematic representation of the strategy used to disrupt the *RAMP3* gene in mice. Exons 2 and 3 of *RAMP3*, including the last 129 amino acids (of 147 total), the stop codon, and the 3′-untranslated region were deleted by homologous recombination. Shown are AvrII (*A*), Kpn (*K*), PvuII (*P*), and SacI (*S*) and P1, P2, P3, and P4 primers used for screening and genotyping. *B*, Southern blot for detection of the wild type and targeted alleles using genomic DNA and the probe fragment depicted in Fig. 2*A*. *C*, genomic PCR for detection of the wild type and targeted alleles using total DNA extracts and the primers depicted in Fig. 2*A*. *D*, quantitative RT-PCR for detection of *RAMP3* mRNA transcripts using total RNA extracted from adult kidneys and the primers and probes described under "Experimental Procedures." *ND*, not detected. *E*, quantitative RT-PCR for detection of *RAMP2* and *calcrl* mRNA transcripts using total RNA extracted from the hearts and lungs of $RAMP3^{-/-}$ adult mice.

we found no significant compensatory increase in the expression of either *RAMP3* or *calcrl* in $RAMP2^{+/-}$ or $RAMP2^{-/-}$ mice compared with wild type controls, demonstrating that the genetic reduction of RAMP2 does not result in a compensatory up-regulation of *RAMP3* or *calcrl* gene expression.

Generation of Mice Lacking the RAMP3 Gene—Homozygous null mice for the *RAMP3* gene were generated by homologous recombination using the targeting strategy shown in Fig. 2*A*. The disrupted allele, which lacks exons 2 and 3 of the *RAMP3* gene (coding for amino acids 19–147 of 147 total amino acids), was detected by Southern blot analysis using a genomic probe fragment located outside the areas of homology (Fig. 2*B*) and by genomic PCR (Fig. 2*C*). The correctly targeted allele was further confirmed by direct sequencing (data not shown). To confirm that the gene targeting effectively disrupted transcription of full-length *RAMP3* mRNA, quantitative reverse transcription-PCR for *RAMP3* mRNA was performed on total RNA isolated from adult kidneys. As expected, $RAMP3^{+/-}$ mice expressed approximately half the wild type *RAMP3* RNA levels (45%, $p < 0.0001$ *versus* wild type), whereas $RAMP3^{-/-}$ mice had no detectable levels of *RAMP3* RNA, thus confirming the complete loss of *RAMP3* expression in adult homozygous mice (Fig. 2*D*). To determine whether genetic deletion of RAMP3 caused a homeostatic compensation in the expression of the *RAMP2* or *calcrl* genes in adult animals, we measured the expression of these genes in the heart and kidneys of $RAMP3^{-/-}$ animals. As shown in Fig. 2*E*, we found no significant compensatory increase in the expression of either *RAMP2* or *calcrl* genes in $RAMP3^{-/-}$ mice compared with wild type controls, demonstrating that the genetic deletion of *RAMP3* does not impact on the regulation of *RAMP2* or of *calcrl* gene expression.

RAMP2 Is Essential for Survival and Normal Fertility—In marked contrast, although the loss of RAMP3 did not affect the survival of *RAMP3*$^{-/-}$ mice to adulthood, we found that genetic loss of RAMP2 caused embryonic lethality, as no *RAMP2*$^{-/-}$ pups were born alive to heterozygote matings. This remarkable contrast in phenotypes (embryonic lethality of *RAMP2*$^{-/-}$ mice *versus* survival of *RAMP3*$^{-/-}$ mice) demonstrates that these two genes have significantly different functions during embryonic development.

Offspring from *RAMP3*$^{+/-}$ matings were born in the expected Mendelian ratio of 1:2:1 (26 *RAMP3*$^{+/+}$:48 *RAMP3*$^{+/-}$: 25 *RAMP3*$^{-/-}$ pups from 15 litters) and had litter sizes comparable with isogenic control matings (6.6 pups/litter for *RAMP3*$^{+/-}$ matings *versus* 6.0 pups/litter for 129S6/SvEv isogenic control matings) (Fig. 3). In contrast, although the ratio of wild type:heterozygote genotypes for viable pups born to *RAMP2*$^{+/-}$ matings was in the expected Mendelian distribution of 1:2 (22 *RAMP2*$^{+/+}$:44 *RAMP2*$^{+/-}$ pups from 29 litters), the average litter size was significantly reduced to 2.1 pups/litter at weaning. This markedly reduced litter size was significantly below the expected litter size of 4.5 pups/litter, which took into consideration the gestational loss of *RAMP2*$^{-/-}$ null embryos (further confirmed by the assessment of litter sizes for *calcrl* heterozygote matings in which there was gestational loss of null embryos (Fig. 3) (18). Thus, although genetic loss of *RAMP3* was dispensable for normal fertility, a modest genetic reduction of *RAMP2* was sufficient to cause marked subfertility, demonstrating that the two genes maintain distinct physiological functions during adulthood.

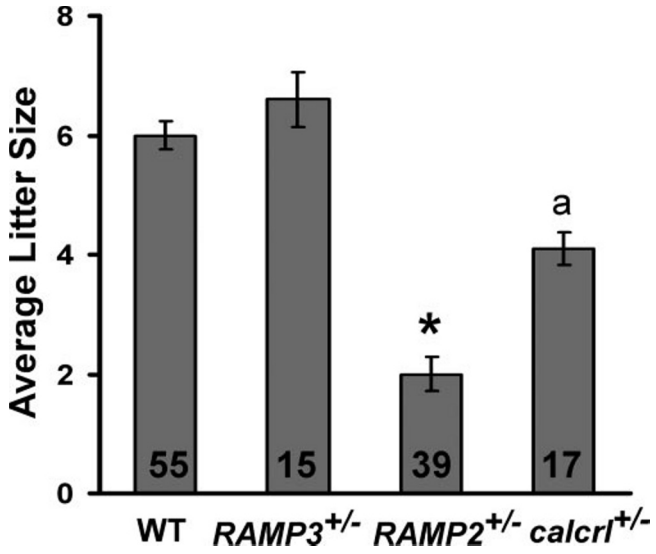

FIGURE 3. **Severely reduced fertility in *RAMP2*$^{+/-}$ mice.** Average litter sizes at weaning resulting from heterozygote matings of the genotype indicated. The *number* at the *bottom* of each bar represents the total number of litters. Litter size of *calcrl*$^{+/-}$ matings is significantly reduced because of the previously reported embryonic lethality of *calcrl*$^{-/-}$ embryos (18). *, $p < 0.05$ by analysis of variance.

Aged RAMP3$^{-/-}$ *Mice Fail to Gain Weight*—*RAMP3*$^{-/-}$ mice survived to adulthood, reproduced, and displayed no obvious phenotypic defects until ~6 months of age. Although the body weights of young *RAMP3*$^{-/-}$ mice did not differ from their wild type controls up to ~6 months of age (Table 1), we noticed that older *RAMP3*$^{-/-}$ mice (9–10 months of age) weighed nearly 9 grams less than age-matched wild type mice (wild type mice weighed 36.1 ± 1.9 g *versus* 27.3 ± 1.1 g for age-matched *RAMP3*$^{-/-}$ mice) (Fig. 4*B*). In contrast, aged *RAMP2*$^{+/-}$ mice did not differ significantly in body weight from their wild type littermates (wild type weighed 28.3 ± 0.5 g *versus* 28.8 ± 0.7 g for age-matched *RAMP2*$^{+/-}$ mice) (Fig. 4*A*). Despite their visibly lean appearance, we found no significant differences in food or water intake in either young or aged *RAMP3*$^{-/-}$ mice compared with their age-matched wild type controls (Table 1). Moreover, *RAMP3*$^{-/-}$ mice, similar to their wild type counterparts, survived to at least 18 months of age with no obvious decline in health. Because *RAMP3* is highly expressed in the proximal tubule of the kidney, we also compared urine volume and kidney function (as determined by protein:creatinine ratio) between *RAMP3*$^{-/-}$ mice and wild type controls and found no obvious differences (Table 1). The body weights, feeding behavior, and kidney function of *RAMP2*$^{+/-}$ mice or *calcrl*$^{+/-}$ mice did not differ from wild type control littermates (Table 1).

Blood Pressure and Heart Rates Are Unaffected in RAMP2$^{+/-}$ *and RAMP3*$^{-/-}$ *Mice*—Because CLR is the best characterized receptor partner for RAMP2 and RAMP3 and because CLR binds to two potent peptide vasodilators (AM and CGRP), we sought to compare the blood pressure and heart rates of *RAMP2*$^{+/-}$ and *RAMP3*$^{-/-}$ mice to those of *calcrl*$^{+/-}$ (18) and wild type mice using a computerized tail cuff system. As shown in Table 1, we found that reduction of *RAMP2* to ~50% of wild type levels and the complete absence of *RAMP3* had no effect on the basal blood pressure of conscious animals compared with wild type mice or with

TABLE 1
Phenotypic analysis of *RAMP2*$^{+/-}$ *and RAMP3*$^{-/-}$ *mice*
Wild type mice were age-, strain-, and gender-matched SvEv129/6.

	Wild type	*RAMP2*$^{+/-}$	*RAMP3*$^{-/-}$	*calcrl*$^{+/-}$
Body weight (4–6 mo., g)	25.8 ± 0.6	27.3 ± 0.8	24.3 ± 0.5	25.6 ± 0.9
Food intake (24 hr, g)	4.6 ± 0.2	4.2 ± 0.2	4.1 ± 0.2	4.5 ± 0.3
Water intake (24 hr, ml)	3.6 ± 0.2	3.7 ± 0.2	3.4 ± 0.1	3.4 ± 0.2
Urine volume (24 hr, ml)	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
Urine protein/creatinine	0.12 ± 0.02	0.12 ± 0.01	0.08 ± 0.01	0.10 ± 0.03
Mean blood pressure (mm Hg)	106 ± 5	105 ± 3	106 ± 5	111 ± 5
Mean heart rate (beats/min)	641 ± 18	635 ± 9	605 ± 10	591 ± 17
Left ventricle/body weight ratio (3–6 months)	4.11 ± 0.22	4.13 ± 0.14	3.98 ± 0.14	3.97 ± 0.17

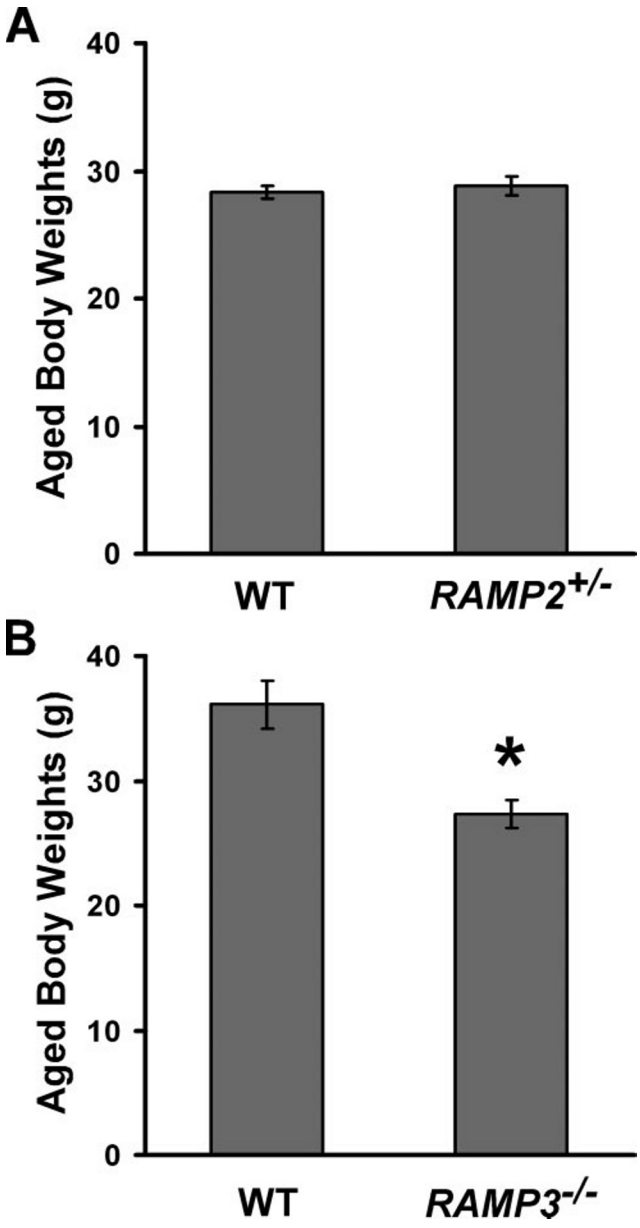

FIGURE 4. **Reduced body weights in aged *RAMP3*$^{-/-}$ mice (but not aged *RAMP2*$^{+/-}$ mice).** *A*, body weights of 9–10-month-old *RAMP2*$^{+/-}$ mice compared with their age-matched isogenic controls (Student's *t* test, $p = 0.6$, $n = 7$ for each group). *B*, body weights of 9–10-month-old *RAMP3*$^{-/-}$ mice compared with their age-matched, isogenic controls (Student's *t* test, $p < 0.001$, $n = 10$ for each group).

calcrl$^{+/-}$ mice. Moreover, we found no overt differences in the heart rates among the genotypes tested (Table 1).

DISCUSSION

In summary, we used gene targeting to generate two independent mouse lines with deletion of either the *RAMP2* or *RAMP3* genes. Gene expression analysis in mice with reduced or absent *RAMP2* levels or a complete lack of *RAMP3* did not reveal any compensatory up-regulation of either *RAMP3* or *RAMP2* gene expression, respectively, supporting our conclusion that there is no functional redundancy at the transcriptional level between *RAMP2* and *RAMP3 in vivo*. We did observe a general trend for significantly *reduced* expression of *RAMP2*, *RAMP3*, and *calcrl* compared with wild type mice in the models we tested (Figs. 1*D* and 2*E*), which is likely reflective of the high sensitivity of these genes to altered physiological homeostasis (11, 12).

Although the biochemical and pharmacological profiles of RAMP2 and RAMP3 appear to overlap for certain GPCRs (CLR, calcitonin, and VIP/VPAC1), our genetic studies demonstrate that the two genes have distinct roles throughout the life of an animal. During embryonic development, *RAMP2*$^{-/-}$ mice failed to survive, whereas *RAMP3*$^{-/-}$ mice appeared normal up to 6 months of age. During adulthood, the loss of *RAMP3* had no apparent effect on fertility. In contrast, a modest genetic reduction of *RAMP2* in heterozygous mice was sufficient to cause a marked reduction in litter size, which is similar to the phenotype we have previously characterized for mice with a genetic reduction of AM (21). Finally, in aged animals, we found that RAMP3 (but not RAMP2) plays an important role in maintaining normal body weight; however, the physiological mechanisms that account for this phenotype have not yet been resolved.

Our studies to address the regulation of blood pressure and heart rate in these mice are consistent with our recent findings demonstrating that genetic alteration of AM peptide levels from 50–140% wild type levels does not affect basal blood pressure. These results are also consistent with another recent study where transgenic overexpression of *mRAMP2* in smooth muscle cells had no effect on basal or induced changes in blood pressure (22). Lu *et al.* (23) have also shown that genetic deletion of α-CGRP does not alter basal blood pressure in mice. Taken together, our results indicate that *in vivo* genetic alteration of *RAMP2*, *RAMP3*, or *calcrl* expression (the receptor signaling components required for transducing the signal of two potent vasodilators, AM and CGRP) does not impact on basal blood pressure regulation in mice. Thus, the use of CLR·RAMP2 or CLR·RAMP3 as pharmacological targets for the treatment of hypertension in humans should be carefully evaluated.

Our most significant data stem from the direct comparison of phenotypes for the *RAMP2* and *RAMP3* gene-targeted mice. Although modest changes in the genetic dosage of the *RAMP2* gene have profound effects on survival and reproduction, complete absence of the *RAMP3* gene seems to have little or no effect on mice until old age. These *in vivo* findings are consistent with the concept that RAMP2 acts to mediate the basal effects of normal GPCR signaling, whereas RAMP3 may become induced under physiological conditions (24) or disease (25) to alter the signaling of GPCRs (10). This concept is further supported by biochemical studies that demonstrate that RAMP3 (but not RAMP2) contains an intracellular PDZ motif capable of binding to *N*-ethylmaleimide-sensitive factor and Na^+/H^+ exchanger regulatory factor to change receptor internalization and trafficking (26, 27). Thus, the continued study of these genetically engineered mouse models under normal conditions and disease states, as well as cell lines derived from them, will provide useful tools for unraveling the functional role of RAMP2 and RAMP3 in modulating GPCR signaling and testing their potential utility as pharmacological targets for the treatment of human disease.

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