Calcitonin gene-related peptide-evoked sustained tachycardia in calcitonin receptor-like receptor transgenic mice is mediated by sympathetic activity

Thomas H. Kunz,1 Michelle Scott,2 Lars M. Ittner,1 Jan A. Fischer,1 Walter Born,1 and Johannes Vogel2

1Research Laboratory, Orthopedic University Hospital Balgrist, and 2Institute of Veterinary Physiology, Vetsuisse-Faculty and Zürich Center of Integrative Human Physiology (ZIHP), University of Zürich, Zürich, Switzerland

Submitted 1 June 2007; accepted in final form 27 July 2007

Calcitonin gene-related peptide (CGRP) and adrenomedullin (AM) are potent vasodilators and exert positive chronotropic and inotropic effects on the heart. Receptors for CGRP and AM are calcitonin receptor-like receptor (CLR)/receptor-activity-modifying protein (RAMP) 1 and CLR/RAMP2 heterodimers, respectively. The present study was designed to delineate distinct cardiovascular effects of CGRP and AM. Thus a V5-tagged CLR was expressed in transgenic mice in the vascular musculature, a recognized target of CGRP. Interestingly, basal arterial pressure and heart rate were indistinguishable in transgenic and in control littermates. Moreover, intravenous injection of 2 nmol/kg CGRP, unlike 2 nmol/kg AM, decreased arterial pressure equally by 18 ± 5 mmHg in transgenic and control animals. But the concomitant increase in heart rate evoked by CGRP was 3.7 times higher in transgenic mice than in control animals. The effects of CGRP in transgenic and control mice, different from a decrease in arterial pressure in response to 20 nmol/kg AM, were suppressed by 2 μmol/kg of the CGRP antagonist CGRP(8-37). Propranolol, in contrast to hexamethonium, suppressed the CGRP-evoked increase in heart rate in both transgenic and control animals. This was consistent with the immunohistochemical localization of the V5-tagged CLR in the superior cervical ganglion of transgenic mice. In conclusion, hypotension evoked by CGRP in transgenic and control mice was comparable and CGRP was more potent than AM. Unexpectedly, the CLR/RAMP2 receptor overexpressed in postganglionic sympathetic neurons of transgenic mice enhanced the positive chronotropic action of systemic CGRP.

calcitonin gene-related peptide; sympathetic nervous system; baroreceptor reflex

α- AND β-CALCITONIN GENE-RELATED PEPTIDE (CGRP) are 37 amino acid neuropeptides derived from separate genes. α-CGRP is widely distributed in the central and peripheral nervous system, whereas β-CGRP is primarily located in enteric nerves and in the pituitary gland (1). α-CGRP and β-CGRP belong to the calcitonin (CT) family of peptides, which includes adrenomedullin (AM) and amylin (16). CGRP and AM are potent vasodilators (2) and exert positive chronotropic and inotropic effects on the heart. Distinct actions of CGRP and AM on the cardiovascular system remain to be delineated. This has been addressed in the present study in transgenic mice expressing a V5-tagged rat CT receptor-like receptor (CLR) under control of a smooth muscle α-actin promoter, e.g., in the vascular musculature.

CGRP immunoreactive nerve fibers terminate in blood vessels, primarily small arteries. They are found at the junction of the adventitia and the media and extend into the muscle layer (11). These observations are consistent with the reported vasodilatory activity of CGRP. In the heart of several mammals, including man, CGRP innervates the atria and, to a limited extent, the ventricles (6, 12, 14). In the atria, CGRP-containing nerve fibres are found in the sino-atrial node, the atrio-ventricular node, and the conduction system. Systemic administration of CGRP in humans and rats has direct positive chronotropic action not blocked by labelatalol (4, 9). Studies on the inotropic action of CGRP on the heart revealed conflicting results (1). In humans, labelatalol suppressed the inotropic action of CGRP, indicating that it is secondary to reflex activation of the sympathetic nervous system (9). In the isolated perfused heart of guinea pigs, CGRP provoked increased contractile force not affected by the β-adrenoceptor antagonist metoprolol (7).

Cardiovascular effects of AM have been studied in conscious sheep and rabbits (8, 17). AM, much like CGRP, provoked decreased blood pressure concomitant with increased heart rate and cardiac output. In a recent study in sheep, AM increased the cardiac sympathetic nerve activity (3). In the isolated rat heart, AM had a positive inotropic effect without affecting the heart rate (19).

Both CGRP and AM interact with the CLR that forms heterodimers with single transmembrane-domain receptor-activity-modifying proteins (RAMP). The three so far identified are RAMP1, -2, and -3, and they determine the ligand selectivity of the CLR (15). CGRP predominantly interacts with CLR/RAMP1, whereas AM is recognized when the CLR is associated with RAMP2 or -3. Thus the responsiveness of a given tissue to AM and/or CGRP depends on the levels of expression of the CLR and the corresponding RAMP.

In the present study, the effects of systemically administered CGRP and AM on arterial blood pressure and heart rate were investigated in transgenic mice expressing a V5-tagged rat CLR (V5-CLR) under the control of a smooth muscle α-actin promoter in the vascular musculature. Littermates were used as control animals. Basal arterial pressure, heart rate, and the vasodilatory response to CGRP and AM were indistinguishable in V5-CLR transgenic and control mice. However, transgenic animals exhibited sustained tachycardia in response to CGRP but not to equal amounts of AM. The effects of CGRP were suppressed by the CGRP(8-37) antagonist and by the β-adrenergic receptor-blocking agent propranolol but not by the ganglion-blocker hexamethonium. Thus CGRP likely increases
the heart rate in transgenic mice through interaction with CLR/RAMP1 CGRP receptors localized on postganglionic sympathetic neurons and independent of the baroreceptor reflex.

**MATERIALS AND METHODS**

**Chemicals.** Rat α-CGRP, α-CGRP(8-37), and AM were purchased from Bachem (Bubendorf, Switzerland). All the peptides were dissolved in isotonic saline (0.9% NaCl) immediately before use. Propranolol was from AstraZeneca (Inderal, 1 mg/ml lyophilized to 10 mg/ml) and hexamethonium from Sigma (Buchs, Switzerland, dissolved in isotonic saline to obtain 25 mg/ml).

**Generation and characterization ofCLR transgenic mice.** Four independent CLR founders were obtained by pronuclear injection of a transgene encoding the V5-CLR (Fig. 1A) into B6D2F1 x B6D2F1 oocytes (10). Founders and offspring were genotyped by PCR analysis of genomic DNA extracted from tail biopsies with transgene-specific forward (5'-GGCCCTGGCCATGGAAGAAGG-3') and reverse (5'-TGGGACCTATGGATGATGAGGAGG-3') primers. PCR products with a predicted size of 880 bp were identified with agarose gel electrophoresis (Fig. 1B). Expression of the V5-CLR in smooth muscle containing tissues was verified by Western blot analysis of extracts of indicated tissues with antibodies to V5 (Invitrogen, Carlsbad, CA) and with monoclonal smooth muscle α-actin specific antibodies (Sigma) and secondary alkaline phosphatase-conjugated antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) (Fig. 1C).

**Surgery and experimental procedure.** All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996), and institutional guidelines and were approved by the Kantonales Veterinäramt Zürich.

**Fig. 1.** Generation of V5 rat calcitonin receptor-like receptor (CLR) transgenic mice. A: the transgene consists of a smooth muscle α-actin promoter, a DNA fragment encoding the signal sequence of the CD33 protein (CD33), a V5 epitope-tag (V5), the cDNA of the rat CLR, and the polyadenylation signal of the bovine growth hormone gene [A(n)]. Arrows labeled P1 and P2 indicate the position of forward and reverse primers used for genotyping the mice. B: agarose gel electrophoresis of PCR-amplified DNA from mouse tail biopsies with the predicted 880-bp transgene-derived product (arrowhead) present in CLR transgenic (CLR<sup>SMoA</sup>) mice but not in control (wt) littermates. C: expression of the V5-CLR (V5) and smooth muscle α-actin (SMoA) in indicated tissues of wt and CLR<sup>SMoA</sup> mice revealed by Western blot analysis.

Four- to five-month-old CLR transgenic mice and control littermates were investigated. Anesthesia was induced with a mixture of 4% halothane, 70% N<sub>2</sub>O, and 26% O<sub>2</sub> and maintained by reducing the inhaled halothane concentration to 1–1.5%. Body temperature was maintained at 37°C using a temperature-controlled heating pad. Catheters were inserted into the left femoral artery and vein for the analysis of pH, Pco<sub>2</sub>, Po<sub>2</sub>, and base excess (pHOx-Plus, Nova Biomedical, Waltham, MA) and for the recording of arterial blood pressure. The arterial catheter was connected to a piezo-electric pressure transducer connected to a bio-potential amplifier (Hugo Sachs Electronics, March, Germany), and the pressure signal was used to calculate the heart rate from peak to peak with a heart rate module (Hugo Sachs Electronics). The signals from the bio-potential amplifier and the heart rate module were digitized, recorded, and analyzed offline with the Power-Lab system (ADInstruments, Spechbach, Germany). After completion of surgery, the anesthesia was changed to intravenous etomidate at an infusion rate of 7–10 μl/min, and 30 min later indicated amounts of peptides were administrated as 50-μl iv bolus injections of corresponding stock solutions. Cardiovascular parameters were monitored for 1 h after the injection of the reagents or until they returned to basal values. Changes in heart rate have been analyzed as area under the curve during the 5 min after the injection of the peptides. The maximal decrease in the arterial blood pressure was also evaluated.

One group of experimental animals was treated with a continuous infusion of propranolol (2 mg·kg<sup>−1·min</sup><sup>−1</sup>) or hexamethonium (5 mg·kg<sup>−1·min</sup><sup>−1</sup>) commencing 30 min before the injection of peptides.

**Collection of sympathetic ganglia.** CLR transgenic mice and control littermates were transcardially perfused with ice-cooled 0.1 M phosphate buffer (pH 7.4), and fixed with 4% paraformaldehyde in PB. The superior cervical ganglion was removed with the carotid bifurcation and immersed in 4% paraformaldehyde in PB; pH 7.4, and fixed with 4% paraformaldehyde in PB for 2 h, cryoprotected in 20% sucrose in PB for 24 h at 4°C, and frozen at −20°C. Serial 10-μm sections of tissue specimens were obtained with a cryostat, mounted on Superfrost plus slides (Menzel-Gläser, Germany), and kept at −20°C until use.

**Immunohistochemistry.** Superior cervical ganglion sections were pretreated with 0.3% H<sub>2</sub>O<sub>2</sub>, washed in PBS, and incubated for 2 h in 1.5% rabbit normal serum or 1.5% goat serum diluted in PBS containing 0.3% Triton X-100. The sections were then incubated with antibodies to tyrosine hydroxylase (Novus Biologicals, Littleton, CO; 1:10,000, 48 h) or to the V5 epitope-tag (Bethyl Laboratories, Montgomery, TX; 1:10,000, 1 h). Subsequently, the sections were incubated with biotinylated goat anti-rabbit or rabbit anti-goat IgG (1:200, 1:10,000, 48 h) or to the V5 epitope-tag (Bethyl Laboratories, Montgomery, TX; 1:10,000, 1 h). Finally, the sections were incubated with biotinylated goat anti-rabbit or rabbit anti-goat IgG (1:200, 2 h) and further processed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), according to the instructions of the manufacturer. The bound antibodies were visualized with 3,3'-diaminobenzidine-tetrahydrochloride (Sigma) as a chromogene. The sections were washed in PBS containing 0.3% Triton X-100 (10 min, 3 times) between incubations.

**Statistics.** Data are presented as means ± SE and analyzed with the GraphPad PRISM 4 Software (version 4.01) using ANOVA and Students t-test for unpaired samples with Bonferroni correction. P values of <0.05 were considered significant.

**RESULTS**

**Basal blood parameters.** pH, Pco<sub>2</sub>, Po<sub>2</sub>, base excess, and hematocrit measured in arterial blood before the administration of drugs and peptides did not differ between control and transgenic mice (Table 1).

**Cardiovascular effects of CGRP.** Arterial blood pressure and heart rate were continuously recorded in anesthetized CLR transgenic mice and control littermates before and after intravenous injection of 2 nmol/kg CGRP. Basal arterial blood pressure and heart rate in CLR transgenic mice were 79 ± 2
mmHg and 361 ± 26 beats/min, respectively, and indistinguishable from 78 ± 5 mmHg and 369 ± 29 beats/min in control animals. Intravenous injection of 2 nmol/kg CGRP in CLR transgenic mice and control animals resulted in a comparable transient decrease in arterial blood pressure that returned to basal levels within 6 min of peptide administration (Fig. 2). However, the concomitant increased heart rate, estimated as area under the curve during the 5 min after injection of CGRP, was 3.7 times higher in CLR transgenic mice than in control animals. Moreover, in CLR transgenic mice, the heart rate remained elevated at 140 ± 8% of basal values at least 30 min after the administration of CGRP. This sustained elevation was not observed in control littermates.

Intravenous injection of 2 μmol/kg of the CGRP(8-37) antagonist had no effect on systemic blood pressure in transgenic and control mice. However, it suppressed the decrease in arterial blood pressure in response to CGRP to a similar extent in control and CLR transgenic animals (Fig. 2). The CGRP evoked increase in heart rate in CLR transgenic mice was also lowered by CGRP(8-37) to values comparable to those of control mice. The CGRP antagonist did not affect the moderate CGRP-induced increase in heart rate observed in control mice. The evidence for CGRP-specific effects on arterial pressure in control and transgenic animals and on the heart rate in CLR transgenic mice was supported by additional observations. Administration of up to 10 times higher amounts of salmon CT than of CGRP did not affect arterial blood pressure or heart rate in CLR transgenic mice and control littermates (not shown). Moreover, 10 times higher amounts of AM than of CGRP were required to obtain a modest decrease in arterial blood pressure in control (not shown) and transgenic mice that was not blocked by CGRP(8-37). Taken together, the results indicate that the higher and prolonged elevation of the heart rate in CLR transgenic mice in response to systemically administered CGRP is mediated by the CLR/RAMP1 CGRP receptor. The sustained CGRP evoked increase in heart rate is unlikely to result from the transient decrease in arterial blood pressure.

**Effect of propranolol and hexamethonium on the action of CGRP.** The enhanced chronotropic action of CGRP was further investigated in CLR transgenic mice and control littermates receiving a constant infusion of the β-adrenergic receptor blocking agent propranolol or the ganglionic blocker hexamethonium (Fig. 3). Treatment of control and CLR transgenic mice with propranolol lowered the arterial blood pressure to 40–50 mmHg. Intravenous injection of 2 nmol/kg CGRP in propranolol-treated mice led to a further transient drop in arterial blood pressure indistinguishable in control and CLR

### Table 1. Basal blood parameters in control and transgenic mice

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<tr>
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<th>pH</th>
<th>Pco2, Torr</th>
<th>Po2, Torr</th>
<th>Base Excess, mM</th>
<th>Hematocrit, %</th>
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<td>wt</td>
<td>7.3±0.1</td>
<td>42.0±5.4</td>
<td>116±26</td>
<td>-2.5±5.5</td>
<td>43.3±3.8</td>
</tr>
<tr>
<td>tg</td>
<td>7.3±0.1</td>
<td>38.7±4.9</td>
<td>116±32</td>
<td>-2.1±5.8</td>
<td>41.0±3.7</td>
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Values are means ± SD. wt, Control mice; tg, calcitonin receptor-like receptor transgenic mice.
transgenic mice and similar to that seen in the absence of propranolol. Importantly, the CGRP evoked increase in heart rate observed in untreated control and CLR transgenic mice was blocked by propranolol, suggesting a CGRP-dependent mechanism upstream of the sino-atrial node independent of the parasympathetic nervous system. Top right: representative tracings of heart rate and arterial blood pressure before and after intravenous injection of CGRP (2 nmol/kg) in wt and tg mice infused with hexamethonium (5 mg·kg⁻¹·min⁻¹). Bottom right: during hexamethonium treatment, the effect of CGRP on heart rate and arterial blood pressure was indistinguishable from that seen in untreated animals (see Fig. 2, left). This suggests that CGRP exerts its effect on postganglionic neurons. Values are means ± SE; n = 5. *P < 0.05 vs. controls.

**DISCUSSION**

The present study introduces transgenic mice that express the rat CLR under the control of a smooth muscle α-actin promoter in vascular tissue and, unexpectedly, also in the superior cervical ganglion. The transgenic mice responded to intravenously injected CGRP with an increased heart rate of higher amplitude and longer duration than control littermates. Interestingly, hypotension provoked by intravenous CGRP was indistinguishable in CLR transgenic and control mice. Apparently, expression of the transgenic rat CLR in the vascular smooth muscle cell layer, a known target of CGRP, did not increase the sensitivity to CGRP or AM. Counterregulation of increased CGRP or AM sensitivity in vascular smooth muscle cells as a result of downregulation of CGRP or AM receptor signaling in CLR transgenic mice or inadequate expression of RAMP1 or -2 cannot be ruled out. The CGRP(8-37) antagonist was ineffective when administered alone. But the hypotensive effect of CGRP was suppressed in both transgenic and control mice by CGRP(8-37). Importantly, the antagonist lowered the increased positive chronotropic action in transgenic animals. In contrast, equal amounts of AM were ineffective in all mice. Thus the observed chronotropic and hypotensive effects in transgenic and wild-type animals are specific for CGRP. Altogether, the results indicate a predominantly baroreflex-independent positive chronotropic action of intravenously administered CGRP in CLR transgenic mice. This is in line with observations in the rat where over 10-fold higher doses of CGRP...
ganglionic sympathetic neurons. This is in accordance with the observed expression of the transgene-derived CLR in the superior cervical ganglion. These findings together with the previously reported inotropic action of CGRP in the dog indicate that CGRP modulates the cardiac output in part through stimulation of myocardial sympathetic activity. The activation of the sympathetic nervous system is a defensive mechanism inhibiting the impact of vasodilation and hypotension.

ACKNOWLEDGMENTS

Present address of L. M. Ittner: Alzheimer’s and Parkinson’s Disease Laboratory, Brain and Mind Research Institute, University of Sydney, Camp-erdown, NSW 2050, Australia.

GRANTS

This study was supported by a grant of the Swiss National Science Foundation to W. Born, a grant from the Zürich Center of integrative Human Physiology (ZIHP) to J. Vogel, the University of Zürich, and the Schweiz-erische Verein Balgrist.

REFERENCES


Fig. 4. Immunoreactive tyrosine hydroxylase (TH) and V5-CLR in sections of the superior cervical ganglion. Top: TH staining in sections from control (TH-wt) and CLR transgenic mice (TH-tg). Note stained cell bodies and unstained nerve fibers (arrowhead). Bottom: V5 staining of ganglia sections of control (V5-wt) and CLR transgenic mice (V5-tg). In CLR transgenic mice, the cell bodies were stained in contrast to the nerve fibers leaving or entering the ganglion (arrowheads). Bar = 50 μm.


