The renin–angiotensin system is an important regulator of arterial blood pressure, electrolyte homeostasis, and water and sodium intake, but it is also involved in other processes, such as tissue regeneration. The renin–angiotensin system is a complex cascade in which precursor peptides are processed by specific enzymes to their active forms. Angiotensinogen is metabolized by renin to the biologically inactive decapeptide angiotensin I (Ang I) that is then cleaved by the angiotensin-converting enzyme to Ang II, the primary active peptide of the renin–angiotensin system.

A homolog of angiotensin-converting enzyme, termed angiotensin-converting enzyme 2, cleaves a single amino acid from the octapeptide Ang II, which produces Ang-(1–7). Because Ang-(1–7) can counter-regulate adverse effects of Ang II, the interest in this peptide significantly increased over the past decade.

In previous work, we identified the G-protein–coupled receptor Mas to be associated with Ang-(1–7)–induced signaling, as for example, genetic deletion of Mas abolishes the binding of Ang-(1–7) to mouse kidney. This binding could also be blocked by cotreatment with d-Ala7-Ang-(1–7), also named A779, a specific Ang-(1–7) antagonist. There is still an intense discussion about the interaction of Ang-(1–7) with the Ang II type 2 receptor. For example, Walters et al described the vasodepressing effect of Ang-(1–7) in AT1 blocker–treated rats can be markedly blocked by PD123319, an AT2 blocker, suggesting that Ang-(1–7) acts via the AT2 receptor.

Furthermore, we and others described that d-Pro7-Ang-(1–7) (d-Pro) blocks Ang-(1–7) effects. Interestingly, the 2 distinct Ang-(1–7) receptor blockers, A779 and d-Pro, reversed the normalizing effects of Ang-(1–7) on systemic
and pulmonary hemodynamics in a model of acute lung injury, but only n-Pro blocked the protection from lung edema and protein leak, whereas A779 restored the infiltration of neutrophils indicating that Ang-(1–7) may act via distinct receptors.9

Therefore, we aimed to identify and quantify a second messenger stimulated by Ang-(1–7) allowing pharmacological confirmation of Mas as a functional heptapeptide receptor, to understand the postulated interaction between AT2 and Ang-(1–7), and to discover the hypothetical second receptor, by working with primary and receptor-transfected cells and in vivo approaches.

Methods

Most of the techniques have been previously described in detail by our group, including culture of primary cells10 and culture and transfection of human embryonic kidney (HEK293) cells.11 A detailed description of techniques, where there is no reference to is provided, is in full detail including cAMP measurement, quantification of protein kinase A (PKA) activity and cAMP response element–binding (CREB) phosphorylation, and measurement of acute hemodynamic effects.

Mice deficient in Mas and MrgD (double knockout) have been generated through breeding of single Mas−/− and MrgD−/− (strain no. 36050, B6.129S1-MrgD−/−Mmnc; The Mutant Mouse Regional Resource Center 8U42DD010924-13, Chapel Hill, NC) knockouts in the animal facilities at University College Cork (UCC), Ireland.

Chemicals and Reagents

Ang-(1–7), d-Ala7-Ang-(1–7), d-Pro7-Ang-(1–7) were from Biosynthetic (Berlin, Germany). Adrenaline, forskolin, HOE 140 (icapitant), 3-isobutyl-1-methylxanthine (IBMX), isoproterenol, Biosynthan (Berlin, Germany). Adrenaline, forskolin, or IBMX were (icapitant), 3-isobutyl-1-methylxanthine (IBMX), isoproterenol, Biosynthan (Berlin, Germany). Adrenaline, forskolin, or IBMX were added for 15 minutes. Then, the cells were lysed in 5% milk/TBS-T buffer for 1 hour, the membrane (Merck KGaA, Darmstadt, Germany) using a Semi Dry Blotter (Schleicher & Schuell, Dassel, Germany). The stimulation with Ang-(1–7) or blockers was carried out as described above. For the test of the adenylyl cyclase (AC) inhibitor (SQ22536), cells were pretreated with 2×10−4 M for 15 minutes, followed by stimulation with Ang-(1–7) at 10−3 M for 15 minutes.

Measurement of cAMP in Cell Lysates

cAMP concentration in cell lysates was determined using Direct cAMP ELISA kit (Enzo Life Sciences Ltd, Exeter, United Kingdom). Briefly, wells of 96-well plate (goat anti-rabbit IgG precoated) were neutralized with 50 μL of Neutralizing Reagent. Next, 100 μL of cell lysate was added, followed by 50 μL of Mouse cAMP antibody conjugate and 50 μL of horseradish peroxidase–conjugated secondary antibody. The plate was incubated for 1 hour at room temperature. The enzymatic reaction was stopped by adding 50 μL of stop solution, and the absorbance at 405 nm was measured immediately. The cAMP concentration was determined from nonlinear standard curve using GraphPad Prism 5.0 software.

Cell Culture Conditions, Transfection, and Stimulation

HEK293 cells were cultured in DMEM medium supplemented with fetal bovine serum (10%), HEPES buffer (1%), sodium pyruvate (1%), glutamine (1%) and maintained under standard conditions (5% CO2, 95% humidity, and 37°C). Cells were cultured in 75-cm² tissue culture flasks and seeded in 48-well plates at density 0.1 N hydrochloric acid with 0.1% Triton by adding 180 μL of wash buffer containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Protein quantification was measured with Pierce BCA Protein Assay Kit according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA).

Assay Kit according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA).

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Isolation and Culture of Primary Cells and Their Stimulation

Primary aortic vascular smooth muscle cells (VSMCs) were isolated from the thoracic aortas of 6- to 8-week-old male C57BL/6 mice and cultured as described previously.10 Kidney mesangial cells (MCs) were isolated from 10- to 12-week-old female mice deficient in Mas,12 MrgD−/− (strain no. 36050, B6.129S1-MrgD−/−Mmnc; The Mutant Mouse Regional Resource Center 8U42DD010924-13, MMRC, Chapel Hill, NC), or in both (generated through cross-breeding of both single knockins in the animal facilities at UCC, Cork, Ireland) and from their age- and sex-matched C57BL/6 control according to the protocol previously described.2 VSMC were used between passage 2 and 3, whereas MC were used at passage 2. Cardiac fibroblasts were isolated from ventricles of adult male C57BL/6 mice as described previously.14 Primary human dermal microvascular endothelial cells were purchased from European Collection of Cell Cultures (Salisbury, United Kingdom). Primary human umbilical vein endothelial cells (HUVEC) were purchased from LONZA BioResearch (Basel, Switzerland). Mouse endothelial cells from cerebral cortex, bEnd.3, were a gift from Dr Siems from FMP (Forschungsinstut fuer Molekulare Pharmakologie, Berlin, Germany). All primary cells were cultured in 75-cm² tissue culture flasks and seeded for cAMP assay in 24-well plates at density of 75,000 cells per well. For protein isolation, cells where cultured in 50-mm dishes until 80% confluence. The stimulation with Ang-(1–7) or blockers was carried out as described above. For the test of the adenylyl cyclase (AC) inhibitor (SQ22536), cells were pretreated with 2×10−4 M for 15 minutes, followed by stimulation with Ang-(1–7) at 10−3 M for 15 minutes.

Western Blot Prism Analyses

Cells were lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). Protein quantification was measured with Pierce BCA Protein Assay Kit according to the manufacturer’s protocol. Twenty-five microgram of protein were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA) using a Semi Dry Blotter (B两地, Luebeck, Germany) containing 4% milk/buffer containing protease and phosphatase inhibitor cocktails. The membrane was incubated for 1 hour at room temperature. The enzymatic reaction was stopped by adding 50 μL of stop solution, and the absorbance at 405 nm was measured immediately. The cAMP concentration was determined from a linear standard curve using GraphPad Prism 5.0 software.
(Cell Signaling Technology) or 1:1000 in 5% milk/TBS-T (Sigma Aldrich) at room temperature for 2 hours. The membranes were developed using the enhanced chemiluminescence–based system (Roche Holding AG, Basel, Switzerland). Densitometric evaluation was performed with ImageJ software (National Institute of Health, New York, NY).

**Protein Kinase A Activity Assay**

The measurement of PKA activity was performed using the PKA Colorimetric Activity Kit (Arbor Assays Headquarters, Ann Arbor, MI) following manufacturer’s instruction. Briefly, HEK293 cells (75,000 per well) were seeded into 48-well plates. On the next day, cells were transiently transfected using PolyFect reagent as described above. For HUVEC cells, 24-well plates (75,000 per well) were used and plated overnight. Sixteen to 20 hours later, the medium containing serum and other supplements was replaced by serum-free medium 1 hour before stimulation. Then, cells were stimulated with Ang(1–7) (10−7 M, 10−8 M, or 10−9 M) for 15 minutes. For experiments with the AC inhibitor (SQ22536), the cells where prestimulated for 15 minutes with 2×10−6 M, followed by stimulation with Ang(1–7) at 10−7 M for 15 minutes. Cells were lysed in 50 μL (HEK293) or 100 μL (HUVEC) of activated cell lysis buffer provided by the kit, containing 1 mmol/L PMSF, 1 μL/mL protease and phosphatase inhibitor cocktail (Sigma) and incubated for 30 minutes on ice. The lysates were transferred to 1.5 mL reaction tubes and centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was used directly for analysis or stored at −80°C till measurement.

For measurement, all samples were diluted 1:10 in kinase assay buffer provided by the Kit. For the measurement, 40 μL of the sample (in triplicates) or standard (in duplicates) were pipetted in to each well. Afterward, 10 μL of ATP was added to each well and incubated for 1.5 hours at 30°C on a shaker. Next, the wells were aspirated and rinsed 4x with wash buffer (1:20 in deionized water). After the final wash, the plate was tapped against clean paper towel to remove any remaining wash buffer. Twenty-five microliter of the goat anti-rabbit IgG horseradish peroxidase and 25 μL of the rabbit phospho PKA substrate antibody were added to each well and incubated at room temperature for 60 minutes with shaking. The wells were aspirated again and rinsed 4x with wash buffer (1:20 in deionized water). After the final wash, the plate was tapped against clean paper towel to remove any remaining wash buffer. Next, 100 μL of the TMB substrate solution were added to each well and incubated for 30 minutes at room temperature. Afterward, 50 μL of stop solution was added to each well and the optical density was measured in a plate reader at 450 nm. The PKA activity (U/mL) was determined from linear standard curve using linear regression from Excel.

**Dual-Luciferase Reporter Assay**

For dual-luciferase reporter assays, HEK293 cells were seeded into 48-well plates (75,000 cells/well). About 24 hours later, 20 cells were transiently transfected with 100 ng eukaryotic expression vectors of AT1, AT2 or Mrg together with 25 ng pNFAT-TA-Luc, (BD Biosciences, Heidelberg, Germany) or pELK-Luc Reporter Vector (Signisign, Santa Clara, CA) luciferase reporter plasmids and 25 ng pK-L-TK (Promega GmbH, Mannheim, Germany) using the protocol described above. Next day, the medium was replaced by serum-free medium 1 hour before stimulation. After 6 hours of stimulation with Ang(1–7) or Ang II (10−7 M and 10−6 M), cells were lysed with 65-μL well passive lysis buffer provided with the Dual Luciferase Reporter Assay kit (Promega GmbH) and incubated on a shaker for 15 minutes at room temperature. The activity for Firefly and Renilla Luciferase was measured with Orion L Microplate Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) according to the manufactures protocol. Briefly, 30 μL of lysed cells were transferred into a white 96-MicroWell plate (Thermo Fisher Scientific), and 100 μL of luciferase assay reagent II was added. After quantifying the firefly luminescence, the reaction was quenched by adding 100 μL of Stop & Glo reagent, and renilla activity was measured. For calculations, the ratio of firefly and renilla luciferase was used.

**mRNA Isolation and Real-Time PCR**

Total cellular RNA was isolated using the NucleoSpin RNA Isolation Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer’s protocol. After isolation, 2000 ng of total RNA was transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis kit (MBI Fermentas, Hanover, MD) and oligo(dT)18 primer. After synthesis, RNase free water was added to the single cDNA to a final volume of 100 μL. Quantification of gene expression was performed by quantitative real-time polymerase chain reaction on the StepOne Real-Time PCR System (Thermo Fisher Scientific) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and specific primers: Mas forward: CCTCCCATCTTCGAGGCTGTA, Mas reverse: GCCCTGGGTGTC-ATTCTATCTT, MrgD forward: TCTAICTGGGTGATGTTAAGACG, MrgD reverse: TCATTAGTA CAGCTTGTAGGCGC, AT2 forward: GAATACCCGTGACCATCA GTCCCT, and AT2 reverse: GGAACCTTAAACACACTGCGGA. Quantitative real-time polymerase chain reaction amplifications were performed in a final volume of 20 μL at 95°C for 15 minutes followed by 40 cycles with denaturation at 95°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 30 s. PCR products were finally subjected to a melting curve analysis. The mRNA levels were quantified with the StepOne analysis software in comparative quantitation mode and normalized to β-actin expression levels. All quantitative real-time polymerase chain reactions were done at least 3× using RNA from independent experiments.

**Measurement of Arterial Pressure and Heart Rate**

The acute mean arterial pressure response to Ang-(1–7) injection was measured by direct cannulation in anesthetized 10- to 14-week-old male mice of wild-type and MrgD-deficient genotype.18 The mice were anesthetized with a combination of ketamine (107 mg/kg body weight) and xylazine (17.1 mg/kg body weight) given intraperitoneally. Body temperature was maintained at 37°C. The right carotid artery was cannulated with PE-10 tubing and connected to a physiological pressure transducer (MEMSCAP, Skopum, Norway), and the mean arterial pressure was continuously recorded with a digital data recorder (Laboratory Chart, ADInstruments Ltd, Oxford, United Kingdom). The heart rate was recorded using mouse needle electrodes (AD Instruments Ltd). After stabilization, Ang-(1–7) (30 μg/kg body weight) was injected in the tail vein. The mean arterial pressure and heart rate were monitored continuously for 30 minutes.

**In Silico Modeling**

The 3-dimensional structure of Ang-(1–7) was prepared by minimizing their nuclear magnetic resonance experimental structures (PDB:2JFP) with AMBER® force field and in water solvent using Schrödinger Macromodel (MacroModel, version 9.7, Schrödinger, LLC, New York, NY). It was further docked (keeping the backbone rigid) with Autodock 4.2,16 into a modeled structure of Mas receptor,17 in which the F112 side chain conformation was modified to create a larger binding site and to express specific binding residue.16,19 The complex was minimized as before. The resulting conformation of Ang-(1–7) was considered the bioactive conformation. Hercules software (Intelligent Pharma S.L., Barcelona, Spain) was applied to superpose PD123319 over Ang-(1–7). PD123319 nonring bonds were treated as flexible (amide bonds set to either 0° or 180°), whereas flexible ring conformations were computed with Corina (Corina, Linux 3.4.6, Molecular Networks GmbH, Erlangen, Germany) using an energy window value of 20 kJ/mol. Ionization states were estimated with Schrödinger Epik (Epik version 2.8017, Schrödinger, LLC, 2009, New York, NY), and Gasteiger charges were assigned to atoms in both molecules as implemented in Open babel.20

**Statistical Analyses**

Data presented are means±SEM. Data were analyzed by Student t test or 1-way and 2-way ANOVA tests accompanied by Bonferroni post hoc test using GraphPad Prism 6.0 (GraphPad Software Inc, San Diego, CA). A P value of <0.05 was considered statistically significant.
Results

Ang-(1–7) Increases Intracellular cAMP in Different Cell Types

Tallant and Clark described an increase in cAMP in VSMC stimulated by Ang-(1–7). Furthermore, Liu et al described the stimulation of intracellular signaling by Ang-(1–7) in glomerular MCs. Their data indicated that the heptapeptide can increase intracellular cAMP. As we aimed to identify the receptors associated with Ang-(1–7) signaling, we initially examined whether cAMP would be an efficient readout to quantify changes in intracellular signaling after Ang-(1–7) stimulation. Ang-(1–7) increased cAMP in a dose-dependent manner in primary MCs with highest efficacy at 10⁻⁷ M and a half-maximal effective concentration (EC₅₀) value of 6.2 nmol/L (Figure 1A). This increase was blocked by the 2 Ang-(1–7) antagonists, A779 and d-Pro, but also by the AT2 receptor blocker PD123319 (Figure 1B). As a variety of papers described the blocking effect of the bradykinin B₂ receptor blocker, icatibant, as well as the inhibitory effect of the NO synthase inhibitor l-NAME, on Ang-(1–7)–mediated signaling, we tested both substances in our primary-cell assay. Although icatibant failed to block an Ang-(1–7)–mediated increase in cAMP, l-NAME inhibited the effects of Ang-(1–7). The heptapeptide could not add to the increased concentration of cAMP reached by l-NAME treatment alone (Figure 1B).

To test the ability of Ang-(1–7) to increase cAMP in other cell types, we stimulated primary cardiac fibroblasts and VSMC with the heptapeptide. Although cardiac fibroblasts did not respond to Ang-(1–7) (Figure 1C), it increased cAMP in VSMC, and the same compounds blocked the effect as described for primary MCs (Figure 1D).

Ang-(1–7) Stimulates the Generation of Intracellular cAMP in Endothelial Cells

Because Ang-(1–7) relaxes vessels in an endothelium-dependent manner, we tested the most commonly used endothelial cell-line, HUVECs, for its response to Ang-(1–7). As described before for other cell-lines, the peptide-mediated increase in intracellular cAMP could be blocked by A779, d-Pro, PD123319, and inhibited by l-NAME (Figure 2A). As shown in Figure 2B, the increase in cAMP was concentration dependent, whereby the EC₅₀ was reached at 13 nmol/L.

![Figure 1](http://hyper.ahajournals.org/)

Figure 1. Intracellular cAMP is increased by angiotensin-(1–7) (Ang-(1–7)) in various cell types. A. Concentration-dependent increase in cAMP level in primary mesangial cells. B. effect of A779, d-Pro7-Ang (1–7) (d-Pro), PD123319, icatibant, and Nω-nitro-l-arginine methyl ester hydrochloride (l-NAME) (all 10⁻⁶ M) on Ang-(1–7) (10⁻⁷ M)–mediated increase in cAMP in mesangial cells, C. lack of increase in intracellular cAMP in cardiac fibroblasts, D. effect of the receptor blockers and l-NAME on Ang-(1–7)–mediated increase in cAMP in primary vascular smooth muscle cells. Data are presented as mean±SEM. ***P<0.001 vs phosphate-buffered saline (PBS), ###P<0.001 vs Ang-(1–7), n=3×3.
Because the concentration of cAMP depends on its generation as well as its degradation, we tested whether the Ang-(1–7)–mediated raise in cAMP is the consequence of increased stimulation of AC or decreased activity of phosphodiesterases. We used Forskolin, an activator of AC, which directly stimulates cAMP level and IBMX, a nonselective phosphodiesterase inhibitor, which inhibits cAMP degradation. As expected, both Forskolin and IBMX led to an increase in intracellular cAMP (Figure 2C). However, Ang-(1–7) could increase cAMP levels stimulated by IBMX but did not further increase the levels reached by Forskolin alone. The additive effect of Ang-(1–7) and IBMX excludes the Ang-(1–7)–mediated raise in cAMP to be a result of phosphodiesterase inhibition.

To finally confirm that Ang-(1–7)–mediated increase in cAMP is AC dependent, we used the specific enzyme inhibitor SQ22536. As shown in Figure 2D, the SQ22536 alone had no effect on cAMP concentration but the inhibitor blunted the Ang-(1–7)–mediated raise in cAMP linking Ang-(1–7) signaling to $G_{\alpha_s}/AC/cAMP$ axis.

Because cAMP initiates a variety of downstream signaling pathways including PKA activation, we tested whether the Ang-(1–7)–mediated increase in cAMP leads to an increased PKA activity. Stimulation of HUVEC with Ang-(1–7) for 15 minutes resulted in a significant increase in PKA activity (Figure 2E).

**Ang-(1–7) Stimulates the Generation of Intracellular cAMP in HEK293 Cells Expressing Either Mas or MrgD**

To test for receptors involved in the Ang-(1–7)–mediated increase in cAMP levels, we used HEK293 cells. First, we tested untransfected cells to exclude that these cells already respond to the heptapeptide. As shown in the left panel of Figure 3A, the heptapeptide had no effect on cAMP levels, confirming HEK293 cells as an ideal tool to measure cAMP in response to Ang-(1–7) in receptor-transfected cells.

We chose 4 different receptors for further tests. First, we tested Mas as Ang-(1–7) has previously shown to be an endogenous ligand for this receptor. Second, Mrg and MrgD receptors...
have been selected, as we showed a significant arachidonic acid release in response to the heptapeptide in Mrg- and MrgD-transfected cells but not in other receptors of the Mas-like family.11 Finally, we also tested the AT2 receptor because PD123319 is thought to be a specific AT2 receptor blocker, and we showed that it blocks the Ang-(1–7)–mediated increase in cAMP (Figures 1B and 1D and 2A). When HEK293 cells were transfected with plasmids for those 4 receptors, Ang-(1–7) stimulation caused a significant increase in intracellular cAMP levels in cells overexpressing Mas or MrgD but not Mrg and AT2 (Figure 3A, right).

To exclude other main signaling pathways for AT2 and Mrg to be activated by Ang-(1–7) involving other G proteins such as \( G_{aq}/11 \) and \( G_{ai} \), we tested activation of these 2 G proteins by measuring activation of transcription factors specifically activated by them. We have chosen nuclear factor for activated T cells, which is known to be coupled to \( G_{aq}/11 \)11 and Elk1 as indicator for \( G_{ai} \) activation.25 Because AT1 receptor is known to signal through \( G_{aq}/11 \) and \( G_{ai} \) after stimulation with Ang II,26 we used this receptor as the positive control. As expected, Ang II increased luciferase production in AT1 receptor expressing cells cotransfected with either \( G_{aq}/11 \), AT1, AT2, or Mrg, and pNFAT-Luc after stimulation with phosphate-buffered saline (PBS), Ang-(1–7), or Ang II (C) luciferase production in HEK293 cells transiently cotransfected with pcDNA3.1, AT1, AT2, or Mrg, and pElk1-Luc after stimulation with PBS, Ang-(1–7), or Ang II. Data are presented as means±SEM. ***P<0.001, **P<0.01 vs related PBS control, n=3×3 (A), n=1×2 (B and C).

We used both Mas and MrgD receptors to investigate in detail their involvement in Ang-(1–7)–mediated signaling. As shown in Figure 4A, Ang-(1–7) stimulation caused a dose-dependent increase in the intracellular cAMP level in Mas-transfected cells with an EC50 of 5.5 nmol/L. Then, we selected the concentration of highest efficacy (10–7 M) for further tests with the previously effective blockers. Figure 4B shows none of the 3 blockers had an effect on cAMP levels in pcDNA3.1- or Mas-transfected cells. However, the increase in cAMP concentration generated by Ang-(1–7) in Mas-transfected cells was blocked by all the 3 blockers (Figure 4B).

Independent experiments were performed on HEK293 cells transfected with MrgD, where Ang-(1–7) also caused a dose-dependent increase in cAMP concentration with an EC50 value of 6.6 nmol/L (Figure 4C). In contrast to Mas-transfected cells, only \( \sigma \)-Pro and PD123319 blocked the Ang-(1–7)–mediated increase in cAMP level, whereas A779 had no significant effect (Figure 4D).

To confirm that the increase in PKA activity seen in HUVEC (Figure 2E) can be associated to Mas and MrgD signaling, we measured activity in response to Ang-(1–7) in Mas- and MrgD-transfected cells. In both receptor-overexpressing cells, the heptapeptide significantly increased PKA activity. This increase was blunted by the AC inhibitor SQ22536 (Figure 4E) confirming cAMP generation as the trigger for PKA activation.

Figure 3. Angiotensin (Ang)-(1–7)–induced signaling is mediated by Mas and MrgD. A, cAMP concentration in untransfected human embryonic kidney (HEK293) cells stimulated with either Ang-(1–7) (10–7 M), A779, \( \sigma \)-Pro7-(Ang-(1–7) (D-Pro) and PD123319 (all 10–6 M; left) and cAMP level in Mas, Mrg, MrgD, and Ang II type 2 (AT2)–transfected HEK293 cells stimulated with Ang-(1–7) (right). B, luciferase production in HEK293 cells transiently cotransfected with pcDNA3.1, AT1, AT2, or Mrg, and pNFAT-Luc after stimulation with phosphate-buffered saline (PBS), Ang-(1–7), or Ang II. C, luciferase production in HEK293 cells transiently cotransfected with pcDNA3.1, AT1, AT2, or Mrg, and pElk1-Luc after stimulation with PBS, Ang-(1–7), or Ang II. Data are presented as means±SEM. ***P<0.001, **P<0.01 vs related PBS control, n=3×3 (A), n=1×2 (B and C).
Ang-(1–7)–Mediated cAMP Generation Is Absent in MCs Derived From Mas/MrgD Knockout Animals

To confirm that Mas and MrgD are the 2 receptors used by Ang-(1–7) to generate cAMP in primary cells, we used MCs derived from double-knockout mice deficient in both receptors. These mice are fertile and without obvious morphological abnormalities. Initially, we examined whether the measured increase in cAMP in MCs is above the threshold to initiate downstream signaling (Figure 1A). Because one of the consequences of cAMP increase can be the activation of the transcription factor cAMP response element–binding protein (CREB),27 we evaluated whether the Ang-(1–7)–mediated increase in cAMP can also induce CREB phosphorylation. Figure 5A and 5B shows that CREB phosphorylation occurred 5 minutes after Ang-(1–7) stimulation and reached control levels within 30 minutes. This induction was blunted by pretreatment with SQ22536 confirming cAMP generation as initial signal for CREB phosphorylation.

Next, we tested whether Ang-(1–7)–mediated increase in cAMP observed in wild-type MCs could be abolished in Mas- or MrgD-deficient cells or cells isolated from Mas/MrgD double knockouts. Deficiency in one of the receptors reduced the Ang-(1–7) signal generated in wild-type cells, whereas the double knockout completely blunted it (Figure 5C).

To exclude upregulation of the remaining receptor and also to confirm that both Mas and MrgD receptors are absent in knockout cells, we investigated the mRNA levels in MCs derived from double knockouts. Figure 5D shows that Mas and MrgD mRNA levels were barely detected in cells from double knockouts. Notably, deficiency in both receptors did not significantly alter AT2 mRNA levels.

Ang-(1–7)–Mediated Acute Hemodynamic Effects Are Blunted in MrgD-Deficient Mice

As we and others showed that vasorelaxant effects of Ang-(1–7) are Mas dependent,8,24 we finally tested whether the genetic deficiency in MrgD would also have an in vivo effect on the hemodynamic properties of Ang-(1–7). Acute bolus infusion of the heptapeptide led to a significant drop in mean arterial blood pressure, whereas no decrease was observed in MrgD knockouts (Figure 5E).

Discussion

The lack in knowing a second messenger for Ang-(1–7) prevented not only the pharmacological confirmation of assumed receptors and the identification of further hypothesized receptors but also the determination of efficacy and potency of a potential receptor/ligand interaction. The need of such second messenger is also illustrated by the fact that 2 publications during the past years intensively investigated phosphorylation of intracellular molecules28 and quantities of intracellular proteins,29 respectively, in response to Ang-(1–7). Although aimed to identify useful molecules for receptor pharmacology, the data published failed to add useful tools to this approach.
Here, we demonstrate that a second messenger, cAMP, is an ideal tool to quantify changes in intracellular signaling mediated by Ang-(1–7). This allowed us to provide final pharmacological evidence that Mas is a functional receptor for Ang-(1–7). More importantly, the use of cAMP as a readout enabled us to screen for other receptors associated with Ang-(1–7) signaling in a hypothesis-based manner. Consequently, these results provide the first experimental proof that MrgD is a second receptor for Ang-(1–7) that has been confirmed under in vivo conditions. The hypothesis that MrgD acts as a receptor for the heptapeptide has been based on different findings including (1) high sequence homology between Mas and MrgD; (2) a description from a Brazilian group that Ala1-Ang-(1–7) but not Ang-(1–7) is a ligand for MrgD, because unpublished modeling of both peptides excludes a decisive effect of the first amino acid on the Gαs activation and thus made it likely that a receptor for Ala1-Ang-(1–7) is also a receptor for Ang-(1–7); and (3) our data that Ang-(1–7) can stimulate arachidonic acid release in MrgD-transfected cells. However, the experiments with Mrg might seed doubts on the latter point, as the relatively moderate arachidonic acid release was not paralleled by an increase in cAMP in Mrg-transfected cells; but association between cAMP and arachidonic acid release warrants further investigation.

We are the first group to establish cAMP as a readout for Ang-(1–7) receptor pharmacology, although there have been indications in the literature for such an association. Beside the work by Tallant and Clark and by Liu et al in primary cells, an article from 2012 showed indirect cAMP involvement in Ang-(1–7) signaling because its modulation of sympathetic activity in the paraventricular nucleus was abolished by an AC inhibitor and a PKA inhibitor.

Furthermore, physiological effects of the heptapeptide and effects in preclinical models also implicate the involvement of cAMP. For example, we demonstrated the dominant effect of Ang-(1–7) on hematopoietic stem cells/progenitors, and there is a significant evidence that cAMP stimulates such processes. Although Mas-deficient animals showed significant effects in preclinical models, they do not show a strong phenotype under physiological conditions except endothelial dysfunction. On the basis of our present data, one could argue that the lack in phenotype is a result of the heptapeptide’s ability to shunt the lack of Mas by using the sequence and signaling-like receptor, MrgD. However, although not intensively phenotyped yet, the double knockouts look normal, are fertile, and show no increased mortality within 12 months. This further supports the hypothesis that Ang-(1–7) is effective especially under disease conditions.
One of the key findings of this study is the exclusion of AT2 as a functional Ang-(1–7) receptor. Our experiments excluded not only activation of G_{i}, by the heptapeptide in AT2-transfected cells and thus an increase in cAMP concentration but also coupling to other major G proteins activated by G-protein–coupled receptors.

Furthermore, our data also provide the identification of the reason for the controversial discussion on Ang-(1–7)/AT2 during the past years. There are only few published papers, which conclude that the effects blocked by PD123319 can also block Mas and MrgD, such a conclusion could be wrong as the blocking effect of PD123319 is not based on AT2 but on both Ang-(1–7) receptors.

This unspecific receptor profile of PD123319 might not be surprising, if we compare its structure with Ang-(1–7). PD123319 structure can be embedded in the molecular volume of the predicted bioactive conformation of Ang-(1–7) bound to Mas and displays chemical similarities to Ang-(1–7), implicating that both molecules fit into the 2 Ang-(1–7) receptors, whereby PD123319 may fail to stimulate intracellular signaling (Figure 5F).

However, identifying PD123319 as a nonspecific AT2 receptor blocker, also requires a careful re-evaluation of papers, which conclude that the effects blocked by PD123319 are mediated via AT2, as we now know that it could also relate to Mas and MrgD.

Taken together, our results correct the view on the renin–angiotensin system (AT2 is not an Ang-(1–7) receptor), expand our understanding of the beneficial angiotensin-converting enzyme 2/Ang-(1–7)/Mas axis to a 2 receptor axis (Mas and MrgD), identify the primary intracellular signaling cascade for the heptapeptide when interacting with its receptors (G_{i}, AC/CAMP leading to an increase in PKA activity and CREB phosphorylation), and force a re-evaluation of >1000 publications concluding that effects blocked by PD123319 are AT2 specific.

**Perspectives**

As Ang-(1–7) has been identified as a protective peptide in cardiovascular diseases, the identification of a second receptor for the peptide allows for the development of tailored drugs (nonpeptidic agonists) stimulating either Mas or MrgD or both receptors depending on their expression/importance in diseases of different pathogenesis.

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**Disclosures**

T. Walther is an inventor of the patent “Use of an Ang-(1–7) receptor agonist in acute lung injury” (Application No. 08016142.5-2107). T. Walther is a scientific advisor of Tarix (Boston, MA). The other authors report no conflicts.

**References**


We discovered MrgD as the second receptor for angiotensin (1–7) (Ang-(1–7)).
Both Mas and MrgD activate adenylyl cyclase initiating an increase in cAMP, consequently activating protein kinase A, and leading to CREB phosphorylation.
We also generated double-knockout mice deficient in both Ang-(1–7) receptors, Mas and MrgD.
We describe them as not having an obvious anatomic/morphological phenotype, being fertile, and not showing increased mortality until 1 year of age.
By identifying PD123319 as a nonspecific Ang II type 2 receptor blocker, which also blocks Mas and MrgD, we now know that effects of the blocker assigned to its interaction with the Ang II type 2 receptor could also relate to the 2 Ang-(1–7) receptors Mas and MrgD.

What Is New?
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What Is Relevant?
- We demonstrate the in vivo relevance of our findings by showing that the lack in Ang-(1–7)/MrgD interaction leads to a much less pronounced vasorelaxant response to Ang-(1–7) in mice deficient in MrgD.

Summary
Our results lead to an expansion and partial revision of the renin–angiotensin system, by identifying a second receptor for Ang-(1–7). We also solved the decade-long controversial discussion of whether Ang II type 2 is a receptor for Ang-(1–7) by excluding this with our experiments and by providing the explanation for the recent confusion.

Novelty and Significance
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- Both Mas and MrgD activate adenylyl cyclase initiating an increase in cAMP, consequently activation protein kinase A, and leading to CREB phosphorylation.
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Anja Tetzner, Kinga Gebolys, Christian Meinert, Sabine Klein, Anja Uhlich, Jonel Trebicka, Oscar Villacañas and Thomas Walther

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