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Original article

Bivalent angiotensin II suppresses oxidative stress-induced hyper-responsiveness of angiotensin II receptor type I

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ABSTRACT

Angiotensin II receptor type I (AT_1R) is a G-protein coupled receptor involved in regulation of body water-electrolyte balance and blood pressure. Oxidative stress promotes AT_1R oligomerization and hyper-responsiveness to its cognate ligand Ang II. In this study, bivalent Ang II, synthesized by linking with aminocaproic acid (Acp) at the N-terminus, was used to induce AT_1R dimerization and hyper-responsiveness in AT_1R-expressed human embryonic kidney (AT_1R-HEK) cells, determined using image correlation spectroscopy (ICS) and by measuring AT_1R-mediated change in intracellular Ca^{2+} concentration, respectively. In addition, ICS was employed to determine distribution pattern of cell-surface AT_1R and its degree of aggregation when stimulated by monomeric (monovalent) and bivalent Ang II under oxidative stress (100 μM H_2O_2) condition in comparison with normal (unoxidized) AT_1R-HEK cells. Bivalent Ang II induced cell-surface AT_1R aggregation/clustering but maintained AT_1R normal signaling response under oxidative stress condition, whereas stimulation by monomeric Ang II or a mixture of monomeric and Acp-modified Ang II (used in the synthesis of bivalent form) resulted in AT_1R hyper-responsiveness. These results suggest that bivalent ligand (viz. Ang II) provides another strategy in the development of novel drugs specifically designed for attenuating aberrant responsiveness of cognate receptor (AT_1R) under pathological (oxidative stress) conditions.

1. Introduction

Angiotensin II receptor type I (AT_1R), the predominant subtype of angiotensin II receptors, plays a significant role in regulating body water–electrolyte balance and blood pressure [1]. AT_1R is a member of G-protein coupled receptor (GPCR) family. Upon binding by its cognate ligand angiotensin II (Ang II), activated AT_1R transduces signals through $G\alpha_q$-IP_3-calcium signaling pathway, leading to activation of renin–angiotensin system (RAS), which promotes vasoconstriction, maintains vascular tone and electrolyte homeostasis. However, AT_1R hyper-function can lead to pathological conditions such as hypertension and atherosclerosis [2].

Western blot analysis of AT_1R on monocyte from normotensive and hypertensive subjects showed that expression levels of AT_1R in both groups are comparable [3]. Interestingly, while normotensive subject expresses monomer receptor, AT_1R from hypertensive group is present in a dimer form [3]. H_2O_2-induced oxidative stress also causes AT_1R dimerization [4]. Using image correlation spectroscopy (ICS), we have previously shown that in AT_1R-HEK cells exposed to 100 μM H_2O_2 for 3 h, cell-surface AT_1R exists in an aggregated state, in which AT_1R becomes hyper-responsive to Ang II stimulation [5]. Thus, the ability to manipulate cell-surface AT_1R oligomerization provides a potential approach for treatment of atherosclerosis and hypertension.

Utilization of synthetic bivalent ligands to target and manipulate GPCR signaling, in particular those involving dimerization, has been reported [6]. A bivalent ligand consists of two monomeric ligands joined together by a spacer molecule in order to provide

Abbreviations: Acp, aminocaproic acid; AT_1R, angiotensin II receptor type I; $[Ca^{2+}]_i$, intracellular calcium ion concentration; CD, cluster density; DA, degree of aggregation; DIEA, *N*,*N*-diisopropyl ethylamine; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; EGTA, ethylene glycol tetraacetic acid; GPCR, G-protein coupled receptor; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; ICS, image correlation spectroscopy; RAS, renin–angiotensin system.

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flexibility for ligand binding to its cognate receptor. Studies, particularly in GPCR families, have demonstrated that synthetic bivalent ligand can significantly alter ligand potency, binding affinity and selectivity, through induction of receptor dimerization and thereby impacting on receptor signaling responses and subsequent cellular activities [7,8]. Interestingly, binding of a dimeric antagonist of gonadotropin-releasing hormone receptor results in receptor dimerization and agonistic signal transduction [9]. The ability of bivalent ligand to induce changes in receptor oligomeric state by bringing monomeric receptors into close proximity has been proposed to account for the unexpected receptor activation and agonistic response, suggesting that physical interactions between receptors are sufficient for induction of receptor activation [10]. Bivalent agomelatine, a potent agonist of melatonin receptor (MR), enhances binding affinity to both melatonin receptor type I (MT_1R) and type II (MT_2R), with a 75-fold increase in selectivity to MT_2R but alters MR pharmacological characteristics from an agonistic to antagonistic response of both MT_1R and MT_2R [11].

There have been a number of studies reporting the use of bivalent ligands to manipulate GPCR pharmacological properties [6,12,13]. Dimerization by transglutaminase-mediated crosslinking of intracellular C-terminus of two adjacent AT_1R molecules enhances receptor-mediated intracellular Ca^{2+} level ($[Ca^{2+}]_i$) [3], however, the synthesis and pharmacological properties of a bivalent Ang II targeting AT_1R have not as yet been investigated. We hypothesized that changes in AT_1R distribution pattern could modulate AT_1R-mediated signaling response and thereby AT_1R-mediated cellular functions. In order to test this notion, we have synthesized bivalent Ang II and examined its ability to perturb oligomeric state of cell-surface AT_1R and its trans-membrane signaling properties. In addition, experiments were conducted to examine these effects in oxidative stress condition, which has been reported to cause clustering of cell surface AT_1R [4,5].

2. Results

2.1. Design and synthesis of bivalent Ang II

In order to synthesize bivalent Ang II, the peptide was subjected to chemical modification. Structure–activity relationship of Ang II for binding to AT_1R indicated that the C-terminus carboxyl group of Ang II is crucial for electrostatic interaction required for high-affinity binding between Ang II and AT_1R [14]. Any modification to the C-terminus would result in severe perturbations to ligand–receptor binding and ability to activate its cognate receptor [15]. In addition to the C-terminus, side-chains of Arg, His and Tyr are also key components for high-binding affinity [16]. Although several amino acid residues in Ang II sequence (viz., Val, Ile, and Pro) are not essential for receptor binding or activation, their side chains are difficult to modify chemically. As the N-terminal Asp residue is not crucial for Ang II function [17], modification at this site should not compromise its physiological properties. Replacement of Ang II N-terminal Asp with sarcosine even resulted in an increase in agonistic activity [18]. Thus we chose Ang II N-terminus as the site to attach a linker to generate the bivalent ligand.

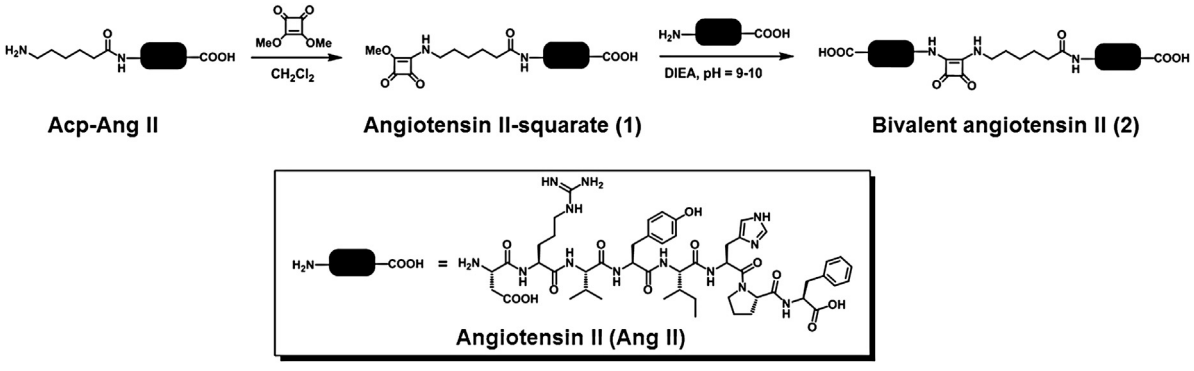
Various types of compounds have been used as linkers in the synthesis of bivalent ligands [6,19]. We opted for aminocaproic acid (Acp), a methylene-based linker which is widely-used in the synthesis of bivalent ligands [20–23], as the chemical structure of Acp allows the ability to extend linker lengths by a simple amide bond formation. Accordingly, Acp was covalently attached to the N-terminus of Ang II to generate Acp-Ang II (Acp-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Acp-Ang II still possesses a functional amine group at the N-terminus, allowing linking to the N-terminus of another Ang II via reaction with the dimethyl squarate (3,4-dimethoxy-3-cyclobutene-1,2-dione) attached to the N-terminus of the second Ang II to yield bivalent Ang II (2) (Scheme 1). Dimethyl squarate was chosen as a linkage for two N-terminal amines because the reaction occurs under mild condition, i.e., in organic solvent or even in aqueous buffer [24–26]. In addition, the conjugation of one amino group could be selectively achieved by adjusting pH [24,27].

2.2. AT_1R-mediated signaling response from stimulation by monovalent and bivalent Ang II in AT_1R-HEK cells under oxidative stress

Transmembrane signaling from binding of Ang II to AT_1R results in $G\alpha_q$ dissociation and phospholipase C activation which sequentially produces secondary messengers diacylglycerol (DAG) and inositol triphosphate (IP_3), generating a variety of cellular responses, such as vasoconstriction, aldosterone release and increase in $[Ca^{2+}]_i$ [28]. As the latter event is an early step in AT_1R-mediated signaling pathway, we monitored $[Ca^{2+}]_i$ in AT_1R-HEK cells preloaded with Ca^{2+}-responsive Indo-1 dye and stimulated by monovalent or bivalent Ang II, then compared the results with oxidatively stressed cells (exposure to 100 μM H_2O_2 for 3 h).

Stimulation of AT_1R-HEK cells by monomeric Ang II, Acp-Ang II, or bivalent Ang II (2) increased $[Ca^{2+}]_i$ 10-fold compared to non-stimulated control cells. (Fig. 1). As expected, Acp-Ang II behaved similarly to unmodified Ang II, but stimulation by Ang II-squarate (1) significantly decreased $[Ca^{2+}]_i$. However, the presence of squarate in the linker of bivalent Ang II (2) did not compromise its ability to increase $[Ca^{2+}]_i$ to the level obtained with Ang II and Acp-Ang II (Fig. 1).

Oxidative-stressed AT_1R-HEK cells, which were induced by H_2O_2 treatment, exhibited AT_1R hyper-function in response to Ang II or to



Scheme 1. Pathway of chemical synthesis of Ang II-squarate (1) and bivalent Ang II (2).

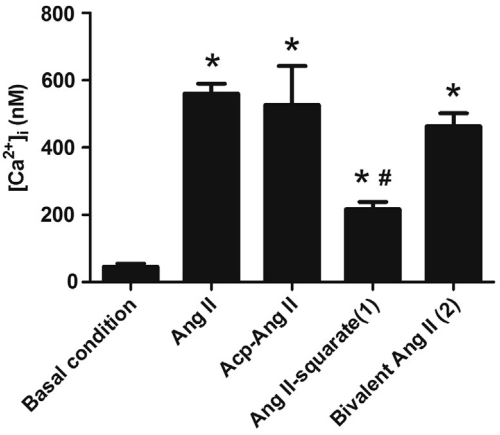


Fig. 1. $[Ca^{2+}]_i$ response of AT_1R-HEK cells induced by Ang II, Acp-Ang II, Ang II-squarate (1) and bivalent Ang II (2). AT_1R-HEK cells (2×10^6 cell/mL HBSS supplemented with 1% (w/v) BSA and 1 mM $CaCl_2$), pre-loaded with Indo1-AM (6 μg/mL), were stimulated with 10 nM Ang II and analogs at 37 °C. Results are presented as $[Ca^{2+}]_i$ from at least three independent experiments performed in triplicate. $^*p < 0.05$ compared with basal condition, $\#p < 0.05$ compared with Ang II stimulation.

a 1:1 mixture of Ang II and Ang II-squarate (1) (Fig. 2), as previously reported [5]. Interestingly, stimulation of oxidative-stressed cells with bivalent Ang II (2) abrogated AT_1R hyper-function, whereas a mixture of Ang II and Ang II-squarate (1), at the same equivalent Ang II concentration as in the bivalent Ang II (2), was still able to stimulate AT_1R hyper-responsiveness (Fig. 2).

2.3. AT_1R distribution pattern induced by monovalent and bivalent Ang II

In order to investigate the underlying mechanism of the inability of bivalent Ang II (2) to induce AT_1R hyper-function in oxidative-stressed AT_1R-HEK cells, we employed ICS [28,29] to characterize the distribution pattern (mean fluorescence intensity, cluster density (CD) and degree of aggregation (DA)) of EGFP-tagged AT_1R on cell surface of oxidized and non-oxidized cells following monovalent Ang II, mixture of Ang II and Ang II-squarate (1), or bivalent Ang II stimulations. Stimulation of oxidative stressed and non-stressed cells by Ang II or a mixture of Ang II and Ang II-squarate (1) significantly increases mean fluorescence intensity of EGFP-tagged AT_1R when compared to unstimulated control cells ($p < 0.05$) (Fig. 3A), indicating an

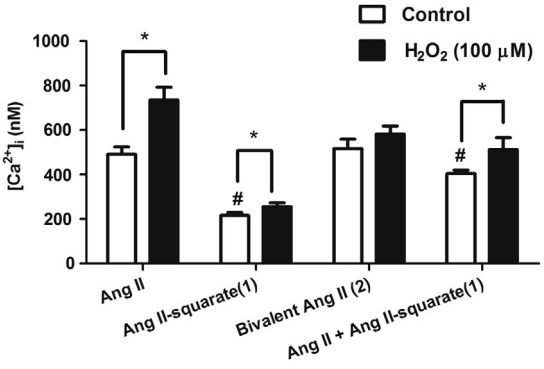


Fig. 2. $[Ca^{2+}]_i$ response of AT_1R-HEK cells induced by monovalent and bivalent Ang II under H_2O_2-mediated oxidative condition. AT_1R-HEK cells (2×10^6 cell/ml) were treated with 100 μM H_2O_2 for 3 h prior to measurement of $[Ca^{2+}]_i$ as descried in legend of Fig. 1. Cells were stimulated by 5 nM indicated peptide (based on Ang II equivalent). $^*p < 0.05$ compared with control group of each tested peptide, $\#p < 0.05$ compared with control group of Ang II stimulation.

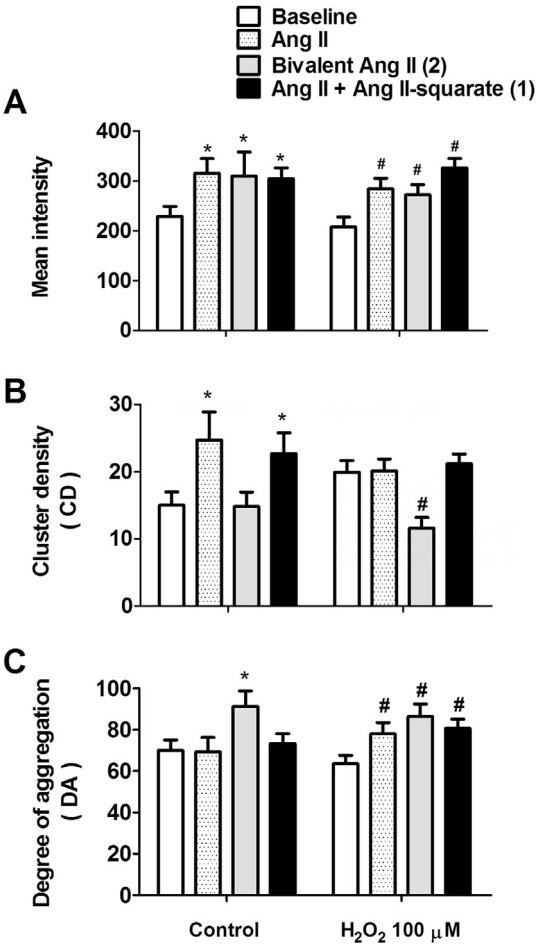


Fig. 3. Cell-surface distribution pattern of EGFP-tagged AT_1R induced by monovalent and bivalent Ang II under H_2O_2-mediated oxidative condition. HEK cells expressing EGFP-tagged AT_1R were treated as described in legend of Fig. 2. EGFP-tagged AT_1R on HEK cell surface, before and after exposure to 5 nM monovalent Ang II, 5 nM (total) monovalent Ang II + Ang II-squarate (1) and 2.5 nM bivalent Ang II (2) were processed for ICS examination as described in Experimental section. A) Mean fluorescence intensity, B) cluster density (CD), C) degree of aggregation (DA). $^*p < 0.05$ compared with non-Ang II stimulation (baseline) group, $\#p < 0.05$ compared with non-Ang II stimulation (baseline) in H_2O_2 pre-treated group.

increase in number of AT_1R at cell surface. These results are in agreement with the previous report of increased mean fluorescence intensity of EGFP-tagged AT_1R on the cell surface upon Ang II stimulation [5].

In non-oxidized cells, both Ang II and mixture of Ang II and Ang II-squarate (1) produced a significant increase in CD values compared to non-stimulated cells ($p < 0.05$), whereas bivalent Ang II (2) had no effect on CD value (Fig. 3B). However, CD values of oxidative stressed cells stimulated by Ang II or Ang II-squarate mixture were comparable to those obtained from non-stimulated cells. This result is in agreement with the previous report [5]. On the other hand, CD value of bivalent Ang II (2)-stimulated oxidized cells is significantly lower than that of non-stimulated cells ($p < 0.05$) (Fig. 3B).

DA values mirrored those of CD, namely. All peptides stimulation caused significant increase in DA of oxidized cells (Fig. 3C). Surprisingly, in non-oxidized cells, a significant increase in DA was observed with bivalent Ang II (2) stimulation ($p < 0.05$) while monomeric Ang II (singly or 1:1 mixture) could not alter DA under control condition (Fig. 3C).

3. Discussion

We have previously demonstrated using ICS that H_2O_2-induced oxidative stress changes the distribution pattern of cell-surface AT_1R resulting in hyper-responsiveness of AT_1R to Ang II stimulation [5]. In this study, we employed bivalent Ang II (2), synthesized by linking N-terminus of two Ang II peptides with aminocaproic acid (Acp), which upon binding to AT_1R-HEK cells should generate AT_1R dimers on cell surface. Unexpectedly, bivalent Ang II (2) stimulation did not induce AT_1R hyper-responsiveness, as deduced from the similarity of $[Ca^{2+}]_i$ in bivalent Ang II (2)-stimulated cells with that of cells stimulated by monomer Ang II, alone or in a 1:1 mixture with Acp-Ang II (Fig. 1).

Nevertheless, ICS showed in non-oxidized cells that bivalent Ang II (2), at the same equivalent of monomeric Ang II concentration, was able to induce aggregation and a decrease in cluster density of cell-surface AT_1R compared to stimulating by monomeric Ang II or non-stimulated control cells (Fig. 3B and C). This evidence solidly supports the abilities of bivalent ligand to induce receptor dimerization [6,12]. On the other hand, 1:1 mixture of Ang II and monovalent Ang II-squarate (1), the chemical components of bivalent Ang II (2), yielded similar cluster density and degree aggregation to those when stimulated by Ang II (Fig. 3B and C). The observations coincided with previous reports on other GPCRs studies, demonstrating that monomeric ligands are unable to cause alteration in distribution or oligomerization of their cognate receptors [30], such as, M3 muscarinic receptor [31], κ opioid receptor [32], δ opioid receptor [33], and adenosine A1 receptor [34].

Under H_2O_2-induced oxidative stress condition, all monovalent forms of Ang II, including Ang II-squarate (1) and 1:1 mixture of Ang II and Ang II-squarate (1), were able to enhance AT_1R responsiveness, manifested by elevation of $[Ca^{2+}]_i$, compared to that of the non-oxidized cells (Fig. 2), in agreement with the previous report [5]. Interestingly, bivalent Ang II (2), did not alter AT_1R responsiveness under H_2O_2-induced oxidative stress condition (Fig. 2). These results can be attributed to the ability of bivalent Ang II (2) to induce clustered AT_1R on cell-surface stemming from the prior exposure to H_2O_2 (Fig. 3C). Taken together, the AT_1R aggregation found in oxidative-stressed cells with bivalent Ang II stimulation resulted from decreasing in CD together with increasing in DA (Fig. 3B, C). This pattern of aggregation was also reported for epidermal growth factor receptor at low temperature [35].

Thereby, under H_2O_2-induced oxidative stress condition, the AT_1R-mediated signaling response, $[Ca^{2+}]_i$ level, was indifferent from the result obtained under control condition. This unique property of the bivalent ligand to retain AT_1R-mediated signaling response under pathological oxidative stress condition might be beneficial for the development of novel angiotensin-based therapeutic for AT_1R implicated diseases.

4. Conclusion

We have demonstrated using ICS that bivalent Ang II is capable of re-organizing AT_1R distribution pattern on HEK cell surface. Remarkably, bivalent Ang II retains normal transmembrane signaling property of AT_1R in pathological (oxidatively stressed) cells, whereas monovalent Ang II enhances AT_1R signaling response (i.e. hyper-responsiveness). Although further investigations into the biochemical and biophysical properties of clustered/aggregated AT_1R are required in order to gain an understanding at the molecular level of their functions under normal physiological and pathological conditions. The unique property of bivalent Ang II to retain normal AT_1R transmembrane signaling under pathological (oxidative stress) conditions indicates another approach in the design and development of therapeutic compounds targeting AT_1R implicated in such diseases as atherosclerosis and hypertension.

5. Experimental

5.1. Materials

Human Ang II (DRVYIHPF; $C_{50}H_{71}N_{13}O_{12}$, MW = 1046.20) and its aminocaproic acid analog Acp-Ang II (Acp-DRVYIHPF; $C_{56}H_{82}N_{14}O_{13}$; MW = 1159.36) were custom synthesized by GL Biochem, Shanghai, China; 3,4-dimethoxy-3-cyclobutene-1,2-dione from Sigma Aldrich, Missouri, USA; Dulbecco's modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS), and Geneticin from Invitrogen, California, USA; fetal bovine serum (FBS), penicillin and streptomycin from HyClone, Thermo Scientific, Utah, USA; Indo-1 acetoxymethyl (Indo-1/AM) from Molecular probes, Invitrogen, California, USA; and EGTA and ionomycin from Calbiochem, New Jersey, USA. Human embryonic kidney cell line containing stably expressed enhanced green fluorescent protein (EGFP)-tagged rat Ang II receptor type I (AT_1R-HEK) was kindly provided by Dr. Tamás Balla, National Institutes of Health, USA [36]. All other commercial chemicals used were of analytical grade.

5.2. Cell culture

AT_1R-HEK cells were cultured in DMEM, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C under humidified atmosphere of 5% CO_2. In order to maintain continuous EGFP-tagged AT_1R expression, every two weeks AT_1R-HEK cells were subjected to selection with 500 μg/ml Geneticin.

5.3. Chemical synthesis

Synthesis of Ang II-squarate (1): Custom synthesized Acp-DRVYIHPF, dissolved in a small volume of dimethyl sulfoxide (DMSO), was added to a solution containing two equivalents of 3,4-dimethoxy-3-cyclobutene-1,2-dione (dimethyl squarate) in dichloromethane (CH_2Cl_2), and pH of the resulting mixture was adjusted a value of 7 by addition of *N*,*N*-diisopropyl ethylamine (DIEA). The reaction mixture was stirred at ambient temperature overnight under an inert N_2 atmosphere. Then, the solution was concentrated under reduced pressure and lyophilized. The product was characterized using high resolution mass spectrometry obtaining a $[M + H]^+$ of 1269.61 (calculated 1267.63 for a mass of $C_{61}H_{85}N_{15}O_{15}$).

Synthesis of bivalent Ang II (2): Synthesized Ang II-squarate (1) was mixed with custom synthesized Ang II in CH_2Cl_2 and pH of the resulting reaction mixture was adjusted to 9–10 by the addition of DIEA. The reaction mixture was stirred overnight at ambient temperature under N_2 atmosphere and then concentrated under reduced pressure and lyophilized. The product was characterized using high resolution mass spectrometry obtaining a $[M + 4H + 4Na]^+$ of 663.44 (calculated 663.75 for a mass of $C_{110}H_{152}N_{28}O_{26}$).

5.4. Intracellular calcium measurement

A fluorometric-based assay was employed to determine intracellular calcium content. Confluent AT_1R-HEK cells were trypsinized and washed twice with HBSS devoid of calcium. Then, cells were resuspended in calcium-free HBSS to at a density of 2×10^6 cell/mL and subsequently incubated with 6 μg/mL Indo1-AM at 37 °C for 60 min. Cells were then washed twice and

resuspended with measuring buffer (HBSS supplemented with 1% (w/v) BSA and 1 mM $CaCl_2$) at 1×10^6 cell/mL. Intracellular baseline calcium level and that upon ligand stimulation were measured as ratio of fluorescence emission at 405 nm and 490 nm, excitation at 338 nm. Fluorescence emission ratio was followed for 3 min after ligand stimulation, unless indicated otherwise. Intracellular calcium content was calculated using the following formula [37]:

$$\left[Ca^{2+}\right]_i = K_d \left[\frac{(R - R_{min})}{(R_{max} - R)}\right] \left[\frac{S_{f2}}{S_{b2}}\right] \quad (1)$$

where, R = ratio of fluorescent emission at 405 nm and 490 nm, K_d = dissociation constant of Indo1-AM, and (S_{f2}/S_{b2}) = fluorescence ratio of 490 nm emission in the absence of Ca^{2+} (presence of 3 mM EGTA) to that at Ca^{2+} saturation (presence of 5 μM ionomycin) [38]. Calcium level induced by ligand stimulation was determined from magnitude of resulting fluorescence signal.

5.5. Image correlation spectroscopy (ICS) and image processing

AT_1R-HEK cells were cultured in cover glass bottom black dish (Electron Microscopy Sciences, Pennsylvania, USA) at a density of 1.5×10^5 cell/plate and observed using a 488 laser line under 60× oil immersion objective, with image acquisition using a 3× digital zoom and Z-stack capture mode at 0.5 μm height for each step (Olympus, FLUOVIEW® FV1000). Final images of 30–40 individual planar cross-section images were reconstructed to obtain a cell-surface distribution pattern of the EGFP-tagged AT_1R. Images captured before and after stimulation by Ang II analog (Ang II, 1:1 mixture of Ang II and Ang II-squarate (1), and bivalent Ang II), were analyzed using image correlation spectroscopy (ICS) [5,28] in order to determine receptor distribution parameters, namely mean fluorescence intensity, cluster density (CD) and degree of aggregation (DA).

5.6. Statistical analysis

Student's *t*-test was used and significant difference is considered at $p < 0.05$. All experimental results are expressed as mean ± S.E.M. from at least three independent experiments performed in triplicate.

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References

[1] C. Oro, H. Qian, W.G. Thomas, Type 1 angiotensin receptor pharmacology: signaling beyond G proteins, Pharmacol. Ther. 113 (2007) 210–226.
[2] P.K. Mehta, K.K. Griendling, Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system, Am. J. Physiol. Cell Physiol. 292 (2007) C82–C97.
[3] S. AbdAlla, H. Lother, A. Langer, Y. el Faramawy, U. Quitterer, Factor XIIIA transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of atherosclerosis, Cell 119 (2004) 343–354.
[4] S. AbdAlla, H. Lother, A. el Massiery, U. Quitterer, Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness, Nat. Med. 7 (2001) 1003–1009.
[5] T. Sungkaworn, Y. Lenbury, V. Chatsudthipong, Oxidative stress increases angiotensin receptor type I responsiveness by increasing receptor degree of aggregation using image correlation spectroscopy, Biochim. Biophys. Acta 1808 (2011) 2496–2500.
[6] I. Berque-Bestel, F. Lezoualc'h, R. Jockers, Bivalent ligands as specific pharmacological tools for G protein-coupled receptor dimers, Curr. Drug Discov. Technol. 5 (2008) 312–318.
[7] E. Hazum, K.J. Chang, H.J. Leighton, O.W. Lever Jr., P. Cuatrecasas, Increased biological activity of dimers of oxymorphone and enkephalin: possible role of receptor crosslinking, Biochem. Biophys. Res. Commun. 104 (1982) 347–353.
[8] F.M. McRobb, I.T. Crosby, E. Yuriev, J.R. Lane, B. Capuano, Homobivalent ligands of the atypical antipsychotic clozapine: design, synthesis, and pharmacological evaluation, J. Med. Chem. 55 (2012) 1622–1634.
[9] P.M. Conn, D.C. Rogers, J.M. Stewart, J. Niedel, T. Sheffield, Conversion of a gonadotropin-releasing hormone antagonist to an agonist, Nature 296 (1982) 653–655.
[10] C.D. Rios, B.A. Jordan, I. Gomes, L.A. Devi, G-protein-coupled receptor dimerization: modulation of receptor function, Pharmacol. Ther. 92 (2001) 71–87.
[11] C. Descamps-Francois, S. Yous, P. Chavatte, V. Audinot, A. Bonnaud, J.A. Boutin, P. Delagrange, C. Bennejean, P. Renard, D. Lesieur, Design and synthesis of naphthalenic dimers as selective MT1 melatoninergic ligands, J. Med. Chem. 46 (2003) 1127–1129.
[12] S.R. George, B.F. O'Dowd, S.P. Lee, G-protein-coupled receptor oligomerization and its potential for drug discovery, Nat. Rev. Drug Discov. 1 (2002) 808–820.
[13] A. Zhang, Z. Liu, Y. Kan, Receptor dimerization – rationale for the design of bivalent ligands, Curr. Top. Med. Chem. 7 (2007) 343–345.
[14] K.H. Hsieh, G.R. Marshall, Role of the C-terminal carboxylate in angiotensin II activity: alcohol, ketone, and ester analogues of angiotensin II, J. Med. Chem. 29 (1986) 1968–1971.
[15] M. de Gasparo, K.J. Catt, T. Inagami, J.W. Wright, T. Unger, International union of pharmacology. XXIII. The angiotensin II receptors, Pharmacol. Rev. 52 (2000) 415–472.
[16] S. Miura, Y.H. Feng, A. Husain, S.S. Karnik, Role of aromaticity of agonist switches of angiotensin II in the activation of the AT1 receptor, J. Biol. Chem. 274 (1999) 7103–7110.
[17] L. Oliveira, C.M. Costa-Neto, C.R. Nakaie, S. Schreier, S.I. Shimuta, A.C. Paiva, The angiotensin II AT1 receptor structure–activity correlations in the light of rhodopsin structure, Physiol. Rev. 87 (2007) 565–592.
[18] M.M. Hall, M.C. Khosla, P.A. Khairallah, F.M. Bumpus, Angiotensin analogs: the influence of sarcosine substituted in position 1, J. Pharmacol. Exp. Ther. 188 (1974) 222–228.
[19] J. Shonberg, P.J. Scammells, B. Capuano, Design strategies for bivalent ligands targeting GPCRs, ChemMedChem 6 (2011) 963–974.
[20] X. Peng, B.I. Knapp, J.M. Bidlack, J.L. Neumeyer, Synthesis and preliminary in vitro investigation of bivalent ligands containing homo- and heterodimeric pharmacophores at mu, delta, and kappa opioid receptors, J. Med. Chem. 49 (2006) 256–262.
[21] W.G. Rajeswaran, Y. Cao, X.P. Huang, M.E. Wroblewski, T. Colclough, S. Lee, F. Liu, P.I. Nagy, J. Ellis, B.A. Levine, K.H. Nocka, W.S. Messer Jr., Design, synthesis, and biological characterization of bivalent 1-methyl-1,2,5,6-tetrahydropyridyl-1,2,5-thiadiazole derivatives as selective muscarinic agonists, J. Med. Chem. 44 (2001) 4563–4576.
[22] J.L. Soulier, O. Russo, M. Giner, L. Rivail, M. Berthouze, S. Ongeri, B. Maigret, R. Fischmeister, F. Lezoualc'h, S. Sicsic, I. Berque-Bestel, Design and synthesis of specific probes for human 5-HT4 receptor dimerization studies, J. Med. Chem. 48 (2005) 6220–6228.
[23] D.J. Daniels, A. Kulkarni, Z. Xie, R.G. Bhushan, P.S. Portoghese, A bivalent ligand (KDAN-18) containing delta-antagonist and kappa-agonist pharmacophores bridges delta2 and kappa1 opioid receptor phenotypes, J. Med. Chem. 48 (2005) 1713–1716.
[24] E.K. Fan, Z.S. Zhang, W.E. Minke, Z. Hou, C. Verlinde, W.G.J. Hol, High-affinity pentavalent ligands of *Escherichia coli* heat-labile enterotoxin by modular structure-based design, J. Am. Chem. Soc. 122 (2000) 2663–2664.
[25] T. Shinada, T. Isida, Y. Ohfune, Recent progress on squaric acid research in bioorganic fields, J. Synth. Org. Chem. 65 (2007) 30–43.
[26] L.F. Tietze, M. Arlt, M. Beller, K.-H. Gl üsenkamp, E. Jähde, M.F. Rajewsky, Anticancer agents, 15. Squaric acid diethyl ester: a new coupling reagent for the formation of drug biopolymer conjugates. Synthesis of squaric acid ester amides and diamides, Chem. Ber. 124 (1991) 1212–1221.
[27] A.H. Schmidt, Reaktionen von Quadratsäure und Quadratsäure-Derivaten, Synthesis 1980 (1980) 961–994.
[28] A. Nohe, N.O. Petersen, Image correlation spectroscopy, Sci. STKE 2007 (2007) l7.
[29] N.O. Petersen, P.L. Hoddelius, P.W. Wiseman, O. Seger, K.E. Magnusson, Quantitation of membrane receptor distributions by image correlation spectroscopy: concept and application, Biophys. J. 65 (1993) 1135–1146.
[30] M.C. Overton, K.J. Blumer, G-protein-coupled receptors function as oligomers in vivo, Curr. Biol. 10 (2000) 341–344.
[31] F.Y. Zeng, J. Wess, Identification and molecular characterization of m3 muscarinic receptor dimers, J. Biol. Chem. 274 (1999) 19487–19497.
[32] B.A. Jordan, L.A. Devi, G-protein-coupled receptor heterodimerization modulates receptor function, Nature 399 (1999) 697–700.

[33] M. McVey, D. Ramsay, E. Kellett, S. Rees, S. Wilson, A.J. Pope, G. Milligan, Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human delta-opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy, J. Biol. Chem. 276 (2001) 14092–14099.
[34] S. Gines, J. Hillion, M. Torvinen, S. Le Crom, V. Casado, E.I. Canela, S. Rondin, J.Y. Lew, S. Watson, M. Zoli, L.F. Agnati, P. Verniera, C. Lluis, S. Ferre, K. Fuxe, R. Franco, Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 8606–8611.
[35] E. Keating, A. Nohe, N.O. Petersen, Studies of distribution, location and dynamic properties of EGFR on the cell surface measured by image correlation spectroscopy, Eur. Biophys. J. 37 (2008) 469–481.
[36] L. Hunyady, A.J. Baukal, Z. Gaborik, J.A. Olivares-Reyes, M. Bor, M. Szaszak, R. Lodge, K.J. Catt, T. Balla, Differential PI 3-kinase dependence of early and late phases of recycling of the internalized AT1 angiotensin receptor, J. Cell Biol. 157 (2002) 1211–1222.
[37] A. Nelemans, Measurement of [Ca^{2+}] in cell suspensions using indo-1, Methods Mol. Biol. 312 (2006) 47–53.
[38] E.B. Puffer, J.K. Pontrello, J.J. Hollenbeck, J.A. Kink, L.L. Kiessling, Activating B cell signaling with defined multivalent ligands, ACS Chem. Biol. 2 (2007) 252–262.