Angiotensin II type 1 receptor-function affected by mutations in cytoplasmic loop CD

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Abstract To explore peptide hormone-induced conformational changes, we attempted to engineer a metal–ion binding site between the cytoplasmic loops CD and EF in the angiotensin II type 1 (AT_1) receptor. We constructed 12 double and six triple histidine mutant receptors, and tested the ability of each mutant and the wild-type to activate inositol phosphate (IP) production with and without $ZnCl_2$. Inhibition by $ZnCl_2$ in the double and triple His mutant receptors was not significant, but these mutations directly decreased the IP production. Systematic analysis of single His mutants demonstrated that the loop CD-mutants displayed 52–74% inhibition of IP production, whereas the loop EF-mutants did not affect IP production. These results indicate that the cytoplasmic loop CD-segment from Tyr^{127} to Ile^{130} is important for $G_{q/11}$ activation by the AT_1 receptor.

Key words: Angiotensin II; G protein coupling; metal–ion binding site; Peptide hormone receptor activation; CD-loop; EF-loop

1. Introduction

The octapeptide hormone angiotensin II (Ang II) is an important regulator of blood pressure, water–electrolyte balance and is an important growth factor for many cell types. Ang II type 1 receptor (AT_1) is necessary and sufficient for regulating most of the known functions attributed to Ang II. The AT_1 receptor contains a seven transmembrane helical structural motif which is common to members of the G protein coupled receptor (GPCR) superfamily. It activates intracellular inositol phosphate (IP) production via coupling to pertussis toxin-insensitive G protein $G_{q/11}$ [1]. The Ang II binding pocket consists of the transmembrane domain and extracellular loops. We have previously reported two salt-bridge interactions which are important for Ang II docking to the receptor and that the interactions of the Tyr^4 and Phe^8 residues of Ang II initiate the AT_1 receptor activation process [2–6]. However, the receptor-G protein interaction sites are poorly understood.

Because the knowledge of the molecular structure of the vast majority of GPCRs is not currently available, indirect experimental methods are currently employed to deduce the structure function relation. A homology model developed by Baldwin [7] and the low resolution structure of visual rhodopsin currently being characterized form the basis for models that can be built for the AT_1 receptor as well as other GPCR membrane proteins [8]. Mutational analysis suggests that key elements involved in Ang II binding and the receptor activation process are similar to many different GPCRs, suggesting that GPCRs may adopt a similar conformation. Therefore, crucial tertiary interactions determined within the G protein binding site for one receptor should be reproducible in another receptor. In one study Thistrup et al. [9] were able to transfer an engineered metal–ion binding site from the NK1 receptor to the κ-opioid receptor demonstrating conservation of the local structure [9,10]. Metal–ion binding sites are suitable for such analysis because the structures of naturally occurring metal binding sites are known and similar sites have been built into other model proteins [11]. These sites are small and geometrically precise interactions required for binding metal ions provide important information regarding the spatial orientation and proximity of amino acids involved [9–12].

Participation of CD- and of EF-loops (also called intracellular loops 2 and 3, respectively) of GPCRs in G protein selection and signal activation is known [13–15]. In addition, mutagenesis, spectroscopy and site-directed spin labeling studies in rhodopsin, a well characterized GPCR, point to motion of TM helices C, F and G as a key element in transmitting signal to the cytoplasmic domain of the receptor [16–19]. The TM helices C and F are in close proximity and are a part of the binding site for the retinal G protein, transducin and suggest that movements of these helices relative to one another are required for transducin activation [20]. Recently, Sheikh et al. [21] demonstrated that a Zn binding site engineered between TM helices C and F in bovine rhodopsin by introduction of His residues at predetermined sites blocked transducin activation by light-activated rhodopsin. In the present study we attempted to move this well defined zinc binding site to the $G_{q/11}$ coupled, peptide hormone receptor, AT_1, which bears only 30% identity with bovine rhodopsin in the TM domain.

2. Materials and methods

2.1. Materials

[Sar^1]Ang II was purchased from Bachem. ^{125}I-[Sar^1,Ile^8]Ang II was purchased from The Peptide Radioiodination Center of Washington State University, Pullman, WA. The specific activity of the ^{125}I-[Sar^1,Ile^8]Ang II was 2200 Ci/mmol. Losartan was a gift from DuPont Merck, Wilmington, DE.

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Abbreviations: Ang II, angiotensin II; GPCR, G protein coupled receptor; AT_1, Ang II type 1 receptor; IP, inositol phosphate; DMEM, Dulbecco's modified minimal essential medium; CPM, counts per minute

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2.2. Mutagenesis and expression of the AT_1 receptor

The synthetic rat AT_1 receptor gene, cloned in the shuttle expression vector pMT-3, was used for expression and mutagenesis, as described earlier [5,6]. Mutants were prepared by Altered Sites® II in vitro mutagenesis system according to the manufacturer's instructions (Promega, Madison, WI). The DNA sequence analysis was done to confirm the mutations. To express the AT_1 receptor protein, 10 μg of purified plasmid DNA/10^7 cells was used in transfection. COS1 cells (American type culture collection, Rockville, MD) cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, were transfected by the DEAE-dextran method. Transfected cells cultured for 72 h were harvested and cell membranes were prepared by the nitrogen parbomb disruption method. The receptor expression was assessed in each case by immunoblot analysis (not shown) and by ^{125}I-[Sar^1,Ile^8]Ang II saturation binding analysis.

2.3. Radioligand binding studies

^{125}I-[Sar^1,Ile^8]Ang II binding experiments were carried out under equilibrium conditions, as described earlier [5,6]. For competition binding studies, membranes expressing the wild-type (WT) receptor or the mutants were incubated at room temperature for 1 h with 300 pM ^{125}I-[Sar^1,Ile^8]Ang II and various concentrations of the agonist or antagonist. All binding experiments were carried out at 22°C in a 250 μl volume. Non-specific binding of the radioligand was measured in the presence of 1 mM ^{127}I-[Sar^1,Ile^8]Ang II. After equilibrium was reached, the binding experiments were stopped by filtering the binding mixture through Whatman GF/C glass fiber filters, which were extensively washed further with binding buffer to wash the free radioligand. The bound ligand fraction was determined from the counts per minute (CPM) remaining on the membrane. Equilibrium binding kinetics were determined using the computer program Ligand®. The K_d values represent the mean ± S.E.M. of three to five independent determinations.

2.4. IP formation studies

Semi-confluent COS1 cells transfected in 60 mm petri dishes were labeled for 24 h with [^{3}H]*myo*-inositol (1.5 μCi/ml), specific activity 22 mCi/mol (Amersham), at 37°C in DMEM containing 10% bovine calf serum. On the day of the functional assay, the labeled cells were washed with HBSS three times and incubated with HBSS containing 10 mM LiCl for 20 min. 10 μM [Sar^1]Ang II, the AT_1 receptor agonist was added and incubated for another 45 min at 37°C. In zinc metal–ion binding experiments, we preincubated with or without 20 mM $ZnCl_2$ for 2 h before washing. 20 mM $ZnCl_2$ was maintained throughout the IP production assay. At the end of incubation, the medium was removed, and total soluble IP was extracted from the cells by the perchloric acid extraction method, as described previously. The amount of [^{3}H]IP eluted from the column was counted and a concentration response curve was generated using iterative non-linear regression analysis (see [5,6]). Results expressed as percentage of IP produced assuming that [Sar^1]Ang II stimulated maximal function.

2.5. Statistical analysis

The results are expressed as the mean ± S.E.M. of two to five independent determinations. The significance differences in measured values were evaluated with an unpaired Student's *t*-test. Statistical significance was set at a level of $P < 0.05$.

3. Results

3.1. Substitution of histidine residues in the proposed interaction site for $G_{q/11}$

The cytoplasmic loops CD and EF participate in the binding sites for the G protein. Fig. 1 shows alignment of CD and EF loop sequences of the AT_1 receptor and bovine rhodopsin. Anchoring the highly conserved DRY sequence indicated that Ala^{129} and His^{132} in the CD loop of AT_1 receptor correspond to Val^{138} and Lys^{141} of the bovine rhodopsin. These residues along with Thr^{251} in the EF loop in rhodopsin formed a Zn binding site when substituted with His [21]. Phe^{239} in the EF-loop of the AT_1 receptor corresponds to Thr^{251} of rhodopsin.

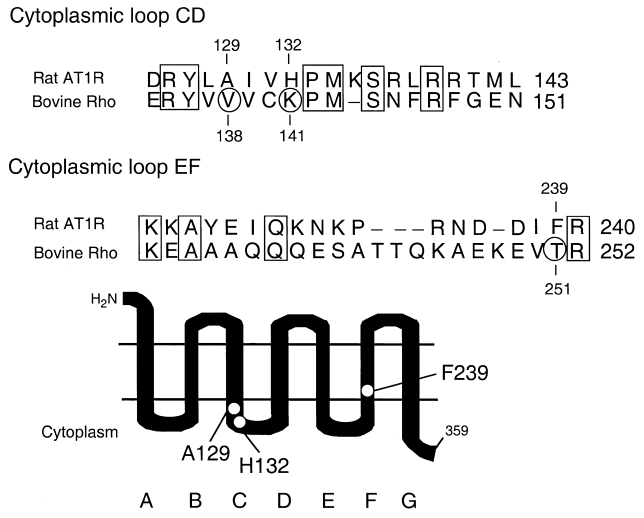

Fig. 1. A secondary structure model of the 359-residue rat AT_1 receptor (AT_1R). The transmembrane segments are putative α-helices forming the ligand pocket. The residues 125–143 of cytoplasmic loop CD and 223–240 of loop EF of rat AT_1 receptor (AT_1R) aligned with the corresponding regions of bovine rhodopsin (Rho) (134–151 of loop CD and 231–252 of loop EF is Rho residue numbering) are shown.

To examine whether the topological arrangement of these residues is conserved between these two GPCRs, we mutated the residues in the AT_1 receptor that correspond to the rhodopsin residues mentioned above. We constructed a triple histidine (A129H/H^{132}/F239H) mutant receptor as a first step for engineering a metal–ion binding site in the G_q-interaction site on the AT_1 receptor, since these residues have been shown to form a Zn binding site in bovine rhodopsin. To extend the mapping study further relevant segments of CD and EF loops were mutagenized. This led to an additional five triple His mutant and 12 double His mutants. These mutants were expressed in COS1 cells, and the expression was measured by immunoblotting (data not shown) and their function was measured by IP-formation (Fig. 1).

3.2. Zinc did not affect IP production in double His and triple His mutant receptors

WT (single histidine, His^{132}), double and triple histidine mutants transiently transfected in COS1 cells yielded comparable levels of cell surface receptors that bound the agonist [Sar^1]Ang II, and the antagonist Dup753 with high affinity. [Sar^1]Ang II stimulated a ≈20-fold increase in IP formation in the WT AT_1 receptor (Fig. 2A). This stimulation was not significantly affected by 1–20 mM $ZnCl_2$ (concentration that inhibited transducin activation by rhodopsin mutants in the same cell system [21]). $ZnCl_2$ alone did not activate IP formation in AT_1 receptor transfected COS1 cells. All of the double and triple histidine mutants activated IP-formation at significantly reduced levels (inhibition was 70 and 59%, respectively) when stimulated with [Sar^1]Ang II. Presence of $ZnCl_2$ did not alter the levels of IP produced in double and triple histidine mutants (2 and 10% reduced, respectively, see Fig. 2A,B) under several different concentrations of $ZnCl_2$.

3.3. Mutations A129H and A129H/H132Q but not F239H alter IP production

To identify the mutation that actually caused the $ZnCl_2$-

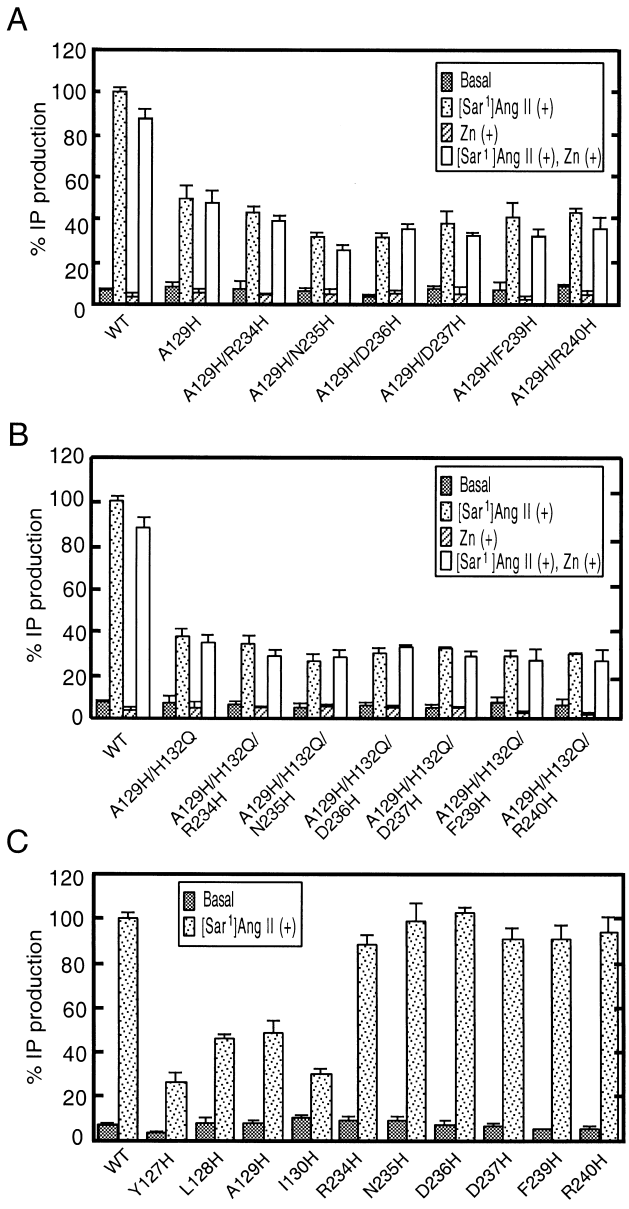

Fig. 2. Basal and [Sar[1]]Ang II-stimulated (10 μM) maximal IP production stimulated by the WT and mutant AT_1 receptors pretreated with or without 20 mM $ZnCl_2$. Basal stimulation of IP accumulation was measured in mock transfected cells ($\approx 1800 \pm 150$ cpm/10^7 cells) and adjusted to 0%. The [Sar[1]]Ang II-stimulated IP accumulation for all mutants shown was calculated considering the stimulation by the WT AT_1 receptor as 100% of ($12\,000 \pm 500$ cpm/10^7 cells). Expression level of each of the mutants was measured by immunoblot analysis. This semi-quantitative method, which is sensitive to measure >two-fold changes in receptor level, indicated that cell surface receptor densities were not significantly different between different mutants examined in this study. Results shown are the mean ± S.E.M. of three independent determinations. $*P < 0.05$ vs. [Sar[1]]Ang II on WT.
←

independent inhibition of IP production, we directly compared A129H, A129H/H132Q and F239H mutant receptors for the ability to bind ligands and stimulate IP production in COS cells. The affinity of the mutant receptors for [Sar[1]]Ang II and Dup753 were similar to that of the WT receptor (Table 1). Inhibition of IP production in the mutant A129H was 52% and was further increased (63%) in the A129H/H132Q mutant receptor (Fig. 2C). The F239H mutation did not affect IP production (Fig. 2B).

3.4. The CD-loop region in the AT_1 receptor is essential for efficient IP formation

To further define the regions that are important for coupling to IP production, we constructed additional mutants – Y127H, L128H, I130H in the CD-loop and R234H, N235H, D236H, D237H, R240H in the EF-loop – in addition to A129H and F239H. K_d of [Sar[1]]Ang II and Dup753 and B_{max} in these mutant receptors were comparable to that of the WT receptor (Table 1). The IP production stimulated by the CD-loop mutants was 52–74% lower compared to the WT (Fig. 2C). The EF-loop mutations (that retain His^{132}, hence are double His mutants) did not affect IP production (Fig. 2B).

4. Discussion

The main finding in this study is the identification of the cytoplasmic CD-loop segment in AT_1 receptor as an important region for efficiently coupling the hormone binding signal

Table 1
Ligand affinities of WT and mutant AT_1 receptors expressed in COS cells

	B_{max} (pmol/mg protein)	K_d (nM)	
		[Sar[1]]Ang II	Dup753
WT (single, H^{132})	4.3 ± 0.3	0.31 ± 0.02	17 ± 1
A129H/H132Q/F239H (double)	3.7 ± 0.6	1.05 ± 0.09	26 ± 1
A129H/H^{132}/F239H (triple)	4.6 ± 0.6	1.15 ± 0.26	21 ± 9
A129H/H132Q	4.1 ± 0.2	0.49 ± 0.15	20 ± 4
Y127H	3.5 ± 0.1	0.32 ± 0.02	16 ± 3
L128H	5.6 ± 0.2	0.35 ± 0.06	16 ± 2
A129H	5.4 ± 0.2	0.34 ± 0.03	23 ± 7
I130H	4.0 ± 0.2	0.42 ± 0.01	20 ± 7
R234H	3.8 ± 0.2	0.31 ± 0.05	17 ± 1
N235H	3.0 ± 0.2	0.48 ± 0.10	19 ± 1
D236H	4.5 ± 0.4	0.83 ± 0.13	12 ± 2
D237H	5.1 ± 0.1	1.18 ± 0.30	15 ± 3
F239H	4.3 ± 0.6	0.94 ± 0.24	25 ± 4
R240H	3.7 ± 0.1	0.91 ± 0.03	17 ± 5

The B_{max} and K_d values represent the mean ± S.E.M. obtained from two of four independent transfection experiments performed in duplicate.

to the activation of $G_{q/11}$ protein leading to IP formation. Substitution of each residue in this segment caused the defect, indicating low tolerance for structural changes. This constraint significantly restricted our attempts to engineer a Zn binding site between the CD-loop and EF-loop. Previously, Ohyama et al. have indicated that the DRY and KSR sequence motifs in the CD-loop are important functions [22]. However, subsequent studies shifted the focus to the functional significance of membrane proximal regions of C-tail and EF-loop [23,24] in the AT_1 receptor. The possibility that the observed defect is because of the His residue substituted instead of isosteric residues in each case cannot be ruled out. But since His substitution at several positions in the EF-loop did not induce similar defects, this explanation appeared unlikely to us. Ridge et al. [17] substituted Cys residues in the CD-loop of bovine rhodopsin to map residues that are critical for transducin activation. Their results indicated that each of the residue between the conserved $Glu^{134}Arg^{135}$ sequence and Pro^{142} inhibited $\approx 70\%$ of transducin activation (see Fig. 1 for comparison). Yang et al. [25] used an identical approach to study the EF-loop residues in rhodopsin and found that the region between Lys^{245} and Arg^{252} when mutated retained $> 80\%$ transducin activation. These observations in rhodopsin are replicated in this study for the AT_1 receptor (Fig. 2). Several different GPCRs have been shown to have an active involvement of loop CD for G protein coupling and of loop EF for signal activation [13–15]. Kobilka et al. [14] suggested that with chimeric α_2- and β_2-adrenergic receptors, the specificity for coupling to the G protein is within loop EF. Kubo et al. [15] concluded that in the muscarinic acetylcholine receptor, the selective coupling of M_1 and M_2 subtypes with different effector systems is due to the loop EF region in these receptors. Wong et al. [26] indicated with chimeric muscarinic acetylcholine/β-adrenergic receptors that the third cytoplasmic loop determines G protein specificity of the receptor. Conservation of these features in the cytoplasmic loops in conjunction with structural and functional features in the TM helices C and F in nearly every GPCR characterized to date has led to a proposed mechanism of signal transduction that is common to all GPCRs [27]. The mechanism predicts rigid body movement of TM helices (C, F and G) inducing conformational changes in the cytoplasmic loops that induce G protein activation by the receptor in response to agonist [27]. How segments on the cytoplasmic domain of different GPCRs interact to select a specific G protein from a pool of structurally similar G proteins remains an intense area of current research.

Tertiary interaction of residues in the cytoplasmic domain of bovine rhodopsin is beginning to be elucidated. Recently Farrens et al. [28] demonstrated that Cys^{139} (in the CD-loop) could be induced to form a disulfide link with a Cys residue introduced at several positions in the EF-loop, indicating their proximity. Engineering of a metal–ion binding site to demonstrate Zn-dependent restrain of activation-induced conformational change in the Sheikh et al. [21] study is an elegant independent confirmation of the proposed spatial proximity of CD and EF loop regions in rhodopsin. We attempted to move this well defined zinc binding site in the present study to the G_q coupled peptide hormone receptor, AT_1 receptor, to demonstrate that geometry of crucial residues for function is similar to that in rhodopsin. The sequence alignments indicated that the AT_1 receptor segments examined here correspond to the CD- and EF-loop regions of bovine rhodopsin (Fig. 1). Effect on IP formation of mutations in these two segments recapitulate the phenotype in rhodopsin indicating that these segments are functionally equivalent to the segments in rhodopsin. Therefore, we anticipated spatial proximity of residues, Ala^{129}, His^{132} and Phe^{239}. We chose these residues for substitution with His in different combinations to create a potential metal–ion binding site. In the presence or absence of $ZnCl_2$ we tested the ability of each of the mutants to activate IP production in COS cells. The results show that Zn did not block IP formation stimulated by these mutants (Fig. 2A). Whether the lack of Zn-inhibition is because of the G protein differences or it indicates differences in the geometry of CD-loop interaction with the EF-loop is unclear. This result is especially important since it is the first report of an attempt to engineer a metal–ion binding site in the G protein binding domain of a hormone receptor. Furthermore, the lack of effect by $ZnCl_2$ may suggest subtle conformational variability between the two receptors compared. The possibility that such differences may be important to distinguish different classes of G proteins ($G_{q/11}$ vs. G_t) needs further study of specific conformation in the AT_1 as well as other GPCRs. This will be the focus of our future studies.

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