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Conformational induction is the key process for activation of the AT_1 receptor

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ABSTRACT

It is currently unclear whether activation of the AT_1 receptor by agonists involves conformational selection or induction. We evaluated the pharmacological properties of wild type and N111G CAM human AT_1 receptors stably expressed in HEK293 cells. Although [Sar^1]-Ang II and Ang IV were full agonists at both receptors, the potency of Ang IV was 280-fold lower at the wild type receptor. [Sar^1, Ile^8]-Ang II was only a full agonist at the N111G CAM AT_1 receptor. [Sar^1]-Ang II and [Sar^1, Ile^8]-Ang II displayed similar high affinity binding to both receptors. In contrast, Ang IV displayed low affinity binding to the wild type and high affinity binding to the N111G CAM AT_1 receptor. Based on these observations we provide strong evidence that conformational induction is the key process for activation of the AT_1 receptor. Only by the creation of CAMs can conformational selection be envisaged to take place.

1. Introduction

The octapeptide angiotensin II (Ang II) is the main effector molecule of the renin–angiotensin system. There are two distinct types of Ang II receptors, AT_1 and AT_2, but the majority of the known physiological actions of Ang II are mediated through the AT_1 receptor [1]. The AT_1 receptor is a member of the heptahelical, G protein-coupled receptor family (GPCR) of receptors. Activation of the receptor leads to stimulation of phospholipase C, inositol phosphate (IP) and calcium mobilisation via the coupling of $G_{q/11}$ proteins [2,3]. The AT_1 receptor is also capable of signalling via G_i, and tyrosine kinase pathways, which are thought to be involved in growth responses [4].

The study of the mechanism of G protein-coupled receptor (GPCR) activation is important since many clinically available drugs act via GPCRs. Both wild type and constitutively active mutant (CAM) GPCRs have given new insights in to receptor activation. The study of CAM adrenoceptors led to the proposal of the most widely accepted model for GPCR activation, the extended ternary complex model (eTCM; [5]). A key aspect of the model is the ability of the receptor to spontaneously undergo reversible isomerization from an inactive (R) to an active (R^*) conformational state in the absence of an agonist. The position of the equilibrium (i.e. the number and proportion of receptors adopting the R^* conformation) defines basal receptor activity. In this model agonists act via conformational selection by preferentially binding to receptors in the R^* state. This results in the stabilisation of R^* which promotes a shift in the equilibrium resulting in increased numbers of R^* receptors. Following this increase, an increase in G protein coupling is observed (R^*G)

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with subsequent effector activation [5]. In the absence of agonists, GPCRs are held in their inactive state by a network of intra-molecular interactions. In CAM GPCRs these interactions are thought to break allowing the receptors to ''relax'' into their active conformations resulting in the observation of increased agonist independent activity. Consequently, CAM GPCRs have been used to study the residues and mechanisms involved in receptor activation. The CAM AT_1 receptors created following substitution of Asn^{111} of the AT_1 receptor with smaller residues such as Gly, Ala and Ser are thought to be formed through the breaking of such constraining intra-molecular bonds between Asn^{111} and residues in TM VII [6–8].

Experimental observations of CAM AT_1 receptors have been cited previously as evidence that the activation of the AT_1 receptor involves conformational selection [6,9]. However, other observations have led to the suggestion that formation of the active conformation involves induction by the agonist in two distinct steps. This induction model was initially proposed by Noda et al. [10] and has been extended more recently by Le et al. [8]. This current model proposes that the Arg^2 residue of Ang II binds to the Asp^{281} residue of the wild type AT_1 receptor and induces a ''pre-activated'' state of the receptor (R′) by causing a conformational change in TM VII that disrupts the constraining intra-molecular bonds between Asn^{111} and residues in TM VII. Following the formation of this ''pre-activated'' state of the receptor, the C-terminal five residues of Ang II bind to the receptor inducing a further transition to the active state (R^*). Intrinsic to this model is the assumption that the N111G CAM AT_1 receptor is a conformational state that mimics the ''pre-activated'' state of the receptor (R′). However, a contradiction in this model was the ability of Ang IV to act as a full agonist at the wild type AT_1 receptor. Since Ang IV lacks Arg^2 and therefore cannot interact with Asp^{281} to induce receptor activation, it demonstrates that full agonist efficacy must reside within residues 3–8 of Ang II.

GPCRs are generally considered to be activated through conformational selection. Following our pharmacological analysis of the peptide ligands [Sar^1]-Ang II, [Sar^1, Ile^8]-Ang II and Ang IV at both the wild type and N111G CAM human AT_1 receptors, we provide strong evidence here that conformational induction is the key process for activation of the AT_1 receptor. Only by the creation of CAMs can conformational selection be envisaged to take place.

2. Methods

2.1. Reagents

Angiotensin II, bacitracin and bovine serum albumin from Sigma (Dorset, UK). ^{125}I-[Sar^1, Ile^8]-Ang II from Perkin-Elmer Life Sciences (Boston, USA). [^{3}H]-Ang II and [^{3}H]-Myo-inositol from Amersham Life Sciences (Buckinghamshire, UK). Ang IV and [Sar^1, Ile^8]-Ang II from Bachem (Essex, UK). Flp-In T-REx Human Embryonic Kidney (Flp-In T-REx HEK293) cells, associated vectors and all cell culture reagents were from Invitrogen (San Diego, USA). Hygromycin B was from Calbiochem (Nottingham, UK). Mirus TransIT^{TM}-293 was from Cambridge Biosciences (Cambridge, UK). QuikChange mutagenesis kit from Stratagene (La Jolla, USA). ABI Prism BigDye Terminator Cycle Sequencing kit from Applied Biosystems (Warrington, UK).

2.2. Site-directed mutagenesis of human AT_1 receptor cDNA

The entire coding region of the human AT_1 receptor (a Kpn I-Not I fragment of 1.5 kb) subcloned into the inducible mammalian expression vector pcDNA5/FRT/TO was used for expression and mutagenesis. The mutation of Asn^{111} to Gly^{111} was performed using the QuikChange mutagenesis kit and confirmed by dideoxy sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit.

2.3. Permanent expression of receptors in Flp-In T-REx HEK293 cells

Cells were cultured in Dulbecco's Modified Eagle Medium without sodium pyruvate, 4500 mg/l glucose and pyroxidone HCl, supplemented with 10% (v/v) foetal calf serum, 1% antibiotic/mycotic mixture, 50 μg/ml gentamicin, 15 μg/ml blastacidin and 4 mM NaOH at 37 °C in a humidified atmosphere of air/CO_2 (19:1). Cells were transfected with 15 μg of cDNA mixture containing either the wild type or mutated human AT_1 pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using Transit-293, according to the manufacturers instructions. Stable expression of human AT_1 receptors in cells was achieved by addition of 200 μg/ml hygromycin B to the medium 3 days after transfection and for all subsequent passages of the cells. Cells were treated with 2 μg/ml doxycycline 48 h prior to assays or preparation of membranes to induce maximal expression of receptors.

2.4. Measurement of inositol phosphate (IP) accumulation

Semi-confluent cells grown in 6 well tissue culture plates were labelled for 48 h with [^{3}H]-myoinositol (3 μCi/ml), at 37 °C in inositol free Dulbecco's Modified Eagle Medium (2 ml) containing 200 mM glutamine and 10% dialysed foetal calf serum. The cells were washed twice with Krebs–Ringer buffer (145 mM NaCl, 5m M KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4) and incubated with Krebs–Ringer buffer (1 ml) containing 10 mM LiCl for 10 min. Subsequently, agonists in Krebs–Ringer buffer (1 ml) containing bovine serum albumin 0.25% (w/v) and 10 mM LiCl were added and incubation continued for a further 12 min at 37 °C. The assay was terminated by addition of 1.5 M perchloric acid (0.5 ml) to the wells and extraction and measurement of total soluble inositol phosphates (IP) undertaken as described previously [11].

2.5. Binding assays

To determine the B_{max} of AT_1 receptor expression cells were grown to confluence in six well tissue culture plates and radioligand–receptor binding with [^{3}H]-Ang II was performed on intact cells as previously described [12]. Competition binding experiments to determine pIC_{50} values were performed on cell membranes by examining the ability of increasing concentrations of peptide ligands to compete with

0.1 nM ^{125}I-[Sar^1, Ile^8]-Ang II binding. The preparation of transfected HEK293 cell membranes and subsequent radioligand–receptor binding assays were undertaken as previously described [6].

2.6. Data analysis

Total radioligand binding (B_{max}) values from assays performed on intact cells were calculated using the equation $1/1 + (k_d/[L])$. Radioligand binding results from assays performed on cell membranes are expressed as a percentage of the control (i.e. specific binding in the absence of any competitor). The binding data were analysed and IC_{50} values determined by non-linear regression analysis using GraphPAD Prism (GraphPAD software Inc., San Diego, USA). Results from inositol phosphate assays were expressed as a percentage of the maximum IP production to a specific agonist. The EC_{50} value for each concentration–response curve was determined using a non-linear regression program and a fixed Hill coefficient of 1.0 using GraphPAD Prism. All results are quoted as the mean ± S.E.M. of three independent experiments unless stated otherwise.

3. Results

The wild type and N111G CAM human AT_1 receptors were stably expressed in Flp-In T-REx HEK293 cells through selection of hygromycin B resistant cells and maximal receptor expression induced by treatment with doxycycline for 48 h. Under these conditions the levels of receptor expression on intact cells were similar, 2146 ± 32 and 1673 ± 54.7 fmol/mg ($n = 3$), for wild type and N111G CAM human AT_1 receptors respectively. The receptors were characterised by examining the ability of peptide ligands to stimulated a rise in intracellular inositol phosphates and to compete with 0.1 nM ^{125}I-[Sar^1, Ile^8]-Ang II binding to membrane preparations.

The accumulation of inositol phosphates was initially measured in cells incubated for 0, 12, 20, 40, 60 and 90 min with Krebs–Ringer buffer containing 10 mM LiCl in the absence of peptide ligands. In cell expressing wild type AT_1 receptors no significant increase in the basal production of inositol phosphates was observed following a 90 min incubation period (1 ± 0.07-fold change compared with time 0). However, in cells expressing the N111G CAM AT_1 receptors production of inositol phosphates increased in a linear fashion as the incubation period increased, reaching a 2.75 ± 0.20-fold change following a 90 min incubation period, thus establishing that these receptors were constitutively active (Fig. 1).

The peptide ligands [Sar^1]-Ang II and Ang IV were both full agonists at the wild type AT_1 receptor with nanomolar and micromolar potencies respectively (Fig. 2 and Table 1). However [Sar^1, Ile^8]-Ang II was observed to be an extremely poor agonist (at 1 μM it produced 1.66 ± 0.7% of a 100 nM angiotensin II response). In stark contrast to the wild type receptor [Sar^1, Ile^8]-Ang II was observed to be a full agonist at the N111G CAM AT_1 receptor (at 1 μM it produced 97.6 ± 1.2% of a 100 nM angiotensin II response) with a potency equivalent to [Sar^1]-Ang II (Fig. 2 and Table 1). Furthermore, Ang IV displayed a 280-fold increase in potency at the N111G CAM AT_1 receptor, resulting in an EC_{50} value that was similar to that observed for [Sar^1]-Ang II at the wild type AT_1 receptor (Fig. 2 and Table 1). However, the potency of [Sar^1]-Ang II did not change (Fig. 2 and Table 1).

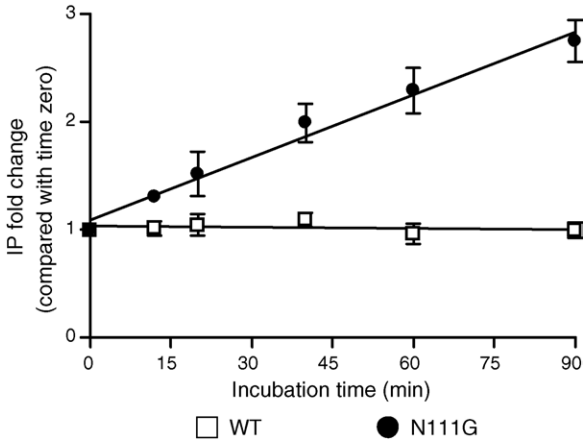

Fig. 1 – Effect of increasing incubation period on basal inositol phosphate production in Flp-In™ T-REx™ HEK293 cell lines expressing either wild type or N111G CAM human AT_1 receptors. Inositol phosphate production following 0, 12, 20, 40, 60 and 90 min incubation of cells with Krebs–Ringer buffer containing 10 mM LiCl was measured. The level of increased basal inositol phosphate production at each time point was calculated as a fold increase of inositol phosphate production at time point 0. Data are expressed as mean ± S.E.M. of results obtained from three independent experiments. In each independent experiment, an average value was derived from triplicate determinations.

Competition binding experiments were undertaken examining the ability of these ligands to compete with 0.1 nM ^{125}I-[Sar^1, Ile^8]-Ang II (Fig. 3 and Table 2). The peptide ligands [Sar^1]-Ang II and [Sar^1, Ile^8]-Ang II competed for single high affinity binding sites with estimated IC_{50} values which were similar between the wild type and N111G CAM AT_1 receptors, 1.6 ± 0.2, 0.9 ± 0.1 and 1.7 ± 0.1, 2.2 ± 0.6 nM, respectively. In contrast, Ang IV competed for a single low affinity binding site to the wild type receptor, with an estimated IC_{50} value of 9044 ± 316 nM, which was in keeping with the micromolar potency observed in the functional assay. Of particular interest was the observation that Ang IV competed for a single high affinity binding site with an estimated IC_{50} value of 2.0 ± 0.3 nM to the N111G CAM AT_1 receptor. This value was similar to the high affinity binding sites observed for [Sar^1]-Ang II and [Sar^1, Ile^8]-Ang II at the wild type and N111G CAM AT_1 receptors and in keeping with the nanomolar potency observed in the functional assay.

4. Discussion

The binding and activation properties of many GPCRs have been explained using receptor theory models based upon the

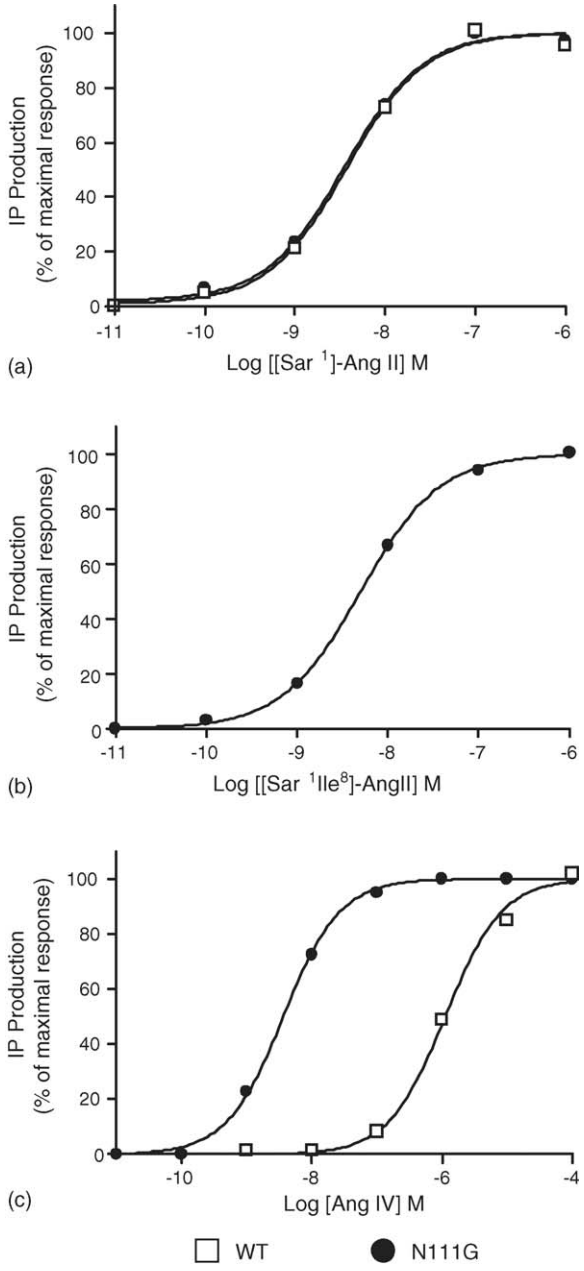

Fig. 2 – [Sar[1]]-Ang II, [Sar[1], Ile[8]]-Ang II and Ang IV concentration-dependent inositol phosphate production in Flp-In™ T-REx™ HEK293 cell lines expressing either wild type or N111G CAM human AT_1 receptors. Inositol phosphate production was measured in cell lines following 12 min stimulation with increasing concentrations of either (a) [Sar[1]]-Ang II, (b) [Sar[1], Ile[8]]-Ang II (no measurable response in cells expressing wild type receptors) or (c) Ang IV. Each point is the mean of triplicate determinations with the mean basal levels subtracted (assay performed in the absence of agonist) from a single experiment, which is representative of three independent experiments. Data are expressed as percentage of maximal agonist response (ascertained by GraphPAD Prism).

concept of ''conformational selection''. Implicit to all such models is the ability of a receptor, in the absence of agonist, to transiently exist in its active state such that there is always a sub-population of active receptors within the total population. Agonists are envisaged to function by preferentially binding to receptors in the active state, thus increasing the proportion of active receptors within the total population by prolonging their existence. Such theories are radically different to the earlier concepts based upon ''conformational induction'' in which the active state of the receptor is induced by the presence of agonist [13].

One of the most widely used and accepted models based upon conformational selection is the extended Ternary Complex Model (eTCM), which was used initially to explain the properties of a constitutively active β_2-adrenoceptor [5]. The eTCM envisages three conformational states of the receptor—the resting state ''R'', an active conformation ''R^*'' and the coupled receptor ''R^*G'' (Fig. 4a). The eTCM contains components from two earlier and more simplistic models, the Two-State Model and the Ternary Complex Model. However, unlike these earlier models, the eTCM separated the isomerisation stage (conversion from the inactive to the active conformation) from the coupling stage (binding of G-protein to active receptor). Implicit to the model is that agonists have a higher affinity for the active R^* state compared with the resting R state. Indeed, Samama et al. [5] demonstrated that there was a direct relationship between the intrinsic activity of agonists and the difference in their affinity for these two receptor states, even at uncoupled receptors. This relationship between affinity and intrinsic activity can be explained by the term ''α'' in the equilibrium scheme in Fig. 4a which directly relates the affinity difference between R and R^* with the equilibrium between R and R^*.

At first glance, the eTCM can be used to explain binding and activation at the AT_1 receptor. For example, substitution of Asn^{111} of the AT_1 receptor with smaller residues such as Gly, Ala and Ser creates constitutively active AT_1 receptors with increased intrinsic activity of partial agonists [10,14]. In particular, substitution of Asn^{111} with Gly (N111G) resulted in the highest levels of constitutive activation and conversion of [Sar[1], Ile[4], Ile[8]]-Ang II and [Sar[1], Ile[8]]-Ang II into full agonists [10]. Therefore the existence of CAM AT_1 receptors suggests that the active conformation of the receptor can exist in the absence of agonist and that its properties can be explained using conformational selection and the eTCM (Fig. 4b). As such, the CAM AT_1 receptors can be considered as representing the AT_1 receptor in its active conformation R^* [6,9].

However, despite the usefulness of conformational selection for explaining the properties of CAM AT_1 receptors, it has been proposed that the AT_1 receptor is in fact activated via a mechanism involving conformational induction of a ''pre-activated'' state R′ [10]. Le et al. [8] have also suggested a model for AT_1 receptor activation using the reaction scheme of Noda et al. [10] involving the ''pre-activated'' state R′. Indeed, two observations from our own data suggest that the eTCM and conformational selection do not explain the binding and activation properties of the wild type angiotensin AT_1 receptor. Firstly, it is clear from our present data and previous studies [6,8,10] that, while [Sar[1]]-Ang II and Ang II display little discrimination between receptor states, Ang IV has a

Table 1 – Receptor expression levels and potency of peptide ligands to stimulate inositol phosphate accumulation for the wild type and N111G CAM AT_1 receptors

	B_{max} (fmol/mg)	[Sar[1]]-Ang II EC_{50} (nM)	[Sar[1], Ile[8]]-Ang II EC_{50} (nM)	Ang IV EC_{50} (nM)
Wild type AT_1 receptor	2146 ± 32	3.3 ± 1.2	ND	1147 ± 70
N111G CAM AT_1 receptor	1673 ± 55	3.3 ± 1.6	5.0 ± 0.7	4.1 ± 0.7

Binding assays were performed on intact cells using 10 nM [^{3}H]-Ang II. The B_{max} values were calculated using the equation $1/1 + (k_d/[L])$. Data are expressed as mean B_{max} ± S.E.M. of results obtained from three independent experiments. Inositol phosphate production was measured in intact cells, following 12 min stimulation with increasing concentrations of peptide ligand. Data, ascertained by GraphPAD Prism, are expressed as mean EC_{50} ± S.E.M. of results obtained from three independent experiments. ND—response too small to measure EC_{50}.

substantially higher affinity for the R^* state over the R state and hence, according to the term "α" in the eTCM, Ang IV should be a better agonist than Ang II. However, the converse obtains since Ang II is a much more potent agonist than Ang IV at the wild type AT_1 receptor. Secondly, the prolonged incubation of cells expressing the wild type AT_1 receptor with LiCl leads to no net accumulation of inositol phosphates (Fig. 1), in stark contrast to Flp-In T-REx HEK293 cells expressing the N111G CAM AT_1 receptor, suggesting that there is little or no spontaneous formation of the R^* state by wild type receptors leading to R^*G signalling complexes in the absence of agonist. While we cannot rule out that there may be a small portion of wild type AT_1 receptors spontaneously adopting the R^* state, this seems highly unlikely. If this were the situation, Ang IV would be expected to preferentially bind to this high affinity conformation and, via conformational selection, shift the equilibrium towards the activated state and thus display high potency similar to that observed for the N111G CAM AT_1 receptor. This was clearly not the case.

Therefore, in agreement with Noda et al. [10] we propose that the active state (R^*) of the AT_1 receptor only exists when stabilised either by a particular mutation, such as N111G, or else "induced" by the binding of agonist. However, Noda et al. [10] envisage an intermediate active receptor conformation R′ and a mechanism that is devoid of conformational selection. In contrast, we have devised a mechanism adapted from the eTCM, whilst incorporating conformational induction. The interpretation of binding and activation data for the wild type and N111G CAM AT_1 receptors are re-interpreted within the context of two radically different reaction schemes (Fig. 4b and c) which are both sub-sets of the eTCM (Fig. 4a). We propose that while the properties of the N111G CAM AT_1 receptors can be explained using the ternary complex model component of the eTCM (Fig. 4b; see Weiss et al. [13] for review), the properties of the wild type receptor must be explained with a classic induction-based scheme requiring three events: (1) ligand binding; (2) induction of the active conformation of the receptor; (3) coupling of G protein with consequent activation of the signalling cascade (Fig. 4c). This induction scheme can be envisaged as a subset of the eTCM in the situation where R^* cannot form in the absence of agonist.

While our data do not allow us to discern the details of the third coupling/activation event, we can clarify the mechanisms underlying both the binding and induction events. It is clear from our present data and a previous study [8] that Ang IV is a full agonist at the wild type receptor despite having both low affinity and potency. This reduced affinity and potency is due to the absence of the arginine residue present at position 2 of Ang II that has been shown to interact with Asp^{281} of the receptor through salt bridge and hydrogen bond interactions [15]. Le et al. [8] suggested that Arg^2 of Ang II was a critical residue in forming the "pre-activated" R′ state of the receptor through its interaction with Asp^{281}, which induced the receptor to adopt the R′ state. However, the reduced potency of Ang IV compared to Ang II can be explained entirely by its reduced affinity at the R state. The consequence of this reduced affinity is that a greater concentration of Ang IV is required to achieve equivalent receptor occupancy compared with Ang II but that, once the equivalent occupancy is achieved, receptor induction and activation are equivalent. Hence the ligand–receptor Arg^2-Asp^{281} interaction is not involved in the induction process as previously reported by Le et al. [8]. At the N111G CAM AT_1 receptor, Ang IV has equivalent affinity to [Sar[1]]-Ang II and Ang II (our data, Le et al. [8,16]) demonstrating that the ligand–receptor Arg^2-Asp^{281} interaction is not required in the binding of the agonist to R^*. Further evidence confirming that the Arg^2 residue of Ang II is involved in receptor affinity, rather than activation, can be seen from the properties of [Sar[1], Ile[8]]-Ang II. This peptide contains Arg^2 but, unlike Ang IV, it is a high affinity antagonist at the wild type receptor in our system.

Since [Sar[1]]-Ang II is a full agonist at the wild type receptor, this loss of receptor activation by [Sar[1], Ile[8]]-Ang II is not due to the sarcosine moiety at position 1, but rather as a result of the Phe^8-Ile substitution. However, since [Sar[1], Ile[8]]-Ang II is a full agonist at the N111G CAM AT_1 receptor, it demonstrates that

Table 2 – Binding affinities (IC_{50}) of peptide ligands for the wild type and N111G CAM AT_1 receptors

	[Sar[1]]-Ang II IC_{50} (nM)	[Sar[1], Ile[8]]-Ang II IC_{50} (nM)	Ang IV IC_{50} (nM)
Wild type AT_1 receptor	1.6 ± 0.2	0.9 ± 0.1	9044 ± 316
N111G CAM AT_1 receptor	1.7 ± 0.1	2.2 ± 0.6	2.0 ± 0.3

Membranes were incubated with the above ligands for 1h in the presence of 0.1 nM ^{125}I-[Sar[1], Ile[8]]-Ang II, prior to separation of bound from free radioligand. Data, ascertained by GraphPAD Prism, are expressed as mean IC_{50} ± S.E.M. of results obtained from three independent experiments.

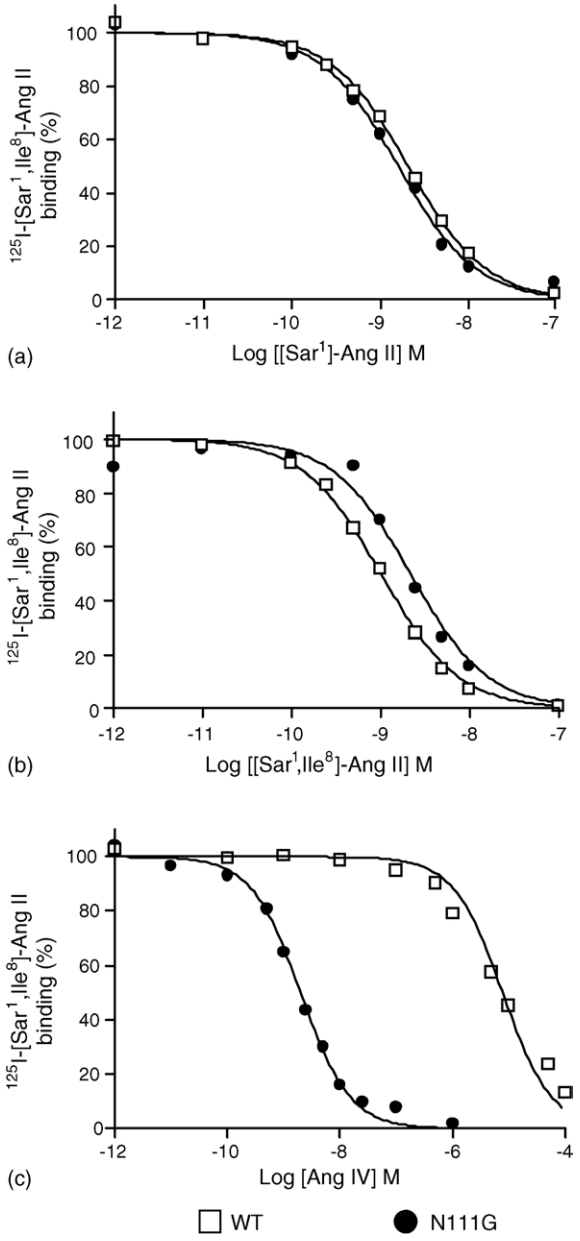

Fig. 3 – Effect of increasing concentrations of (a) [Sar^1]-Ang II, (b) [Sar^1, Ile^8]-Ang II or (c) Ang IV on specific binding of ^{125}I-[Sar^1, Ile^8]-Ang II to membranes derived from Flp-In™ T-REx™ HEK293 cell lines expressing either wild type or N111G CAM human AT$_1$ receptors. Membranes were incubated with increasing concentrations of the above ligands for 1 h in the presence of 0.1 nM ^{125}I-[Sar^1, Ile^8]-Ang II, prior to separation of bound from free radioligand. Each point is the mean of duplicate determinations from a single experiment, which is representative of three independent experiments. Data are expressed as percentage specific ^{125}I-[Sar^1, Ile^8]-Ang II binding in the absence of any competitor.

the Phe^8 side chain is critical for the induction event rather than the coupling/activation event as previously reported [10].

Therefore, these data can be explained through an adaptation of the eTCM in which we envisage that there is no spontaneous formation of R* by the AT$_1$ receptor unless enabled by a mutation such as the N111G. Ironically, this adaptation of the eTCM results in the replacement of conformational selection by conformational induction as the principal mechanism at the wild type receptor. The model allows us to identify four separate interactions a–d between Ang II and the receptor (Fig. 5). Interaction (a) represents the affinity-enhancing component of the docking interaction which requires Arg^2 of the ligand and Asp^{281} of the receptor's R state. Since [Sar^1]-Ang II and [Sar^1, Ile^8]-Ang II can form this interaction they both have high affinity for R while Ang IV, which lacks Arg^2, has low affinity. However, this ligand–receptor Arg^2-Asp^{281} interaction is not required for peptide interaction with the R* state, since all three ligands have equivalent high affinity for the N111G CAM AT$_1$ receptor. Interaction (b) results in the low affinity interaction between the receptor and residues 3–7 of Ang II and its related peptides, as well as with the α-COO$^-$ group of residue 8 that is believed to interact with Lys^{199} of the receptor [10,17]. Hence this low affinity binding can be obtained for peptides lacking residues 1 and 2 (e.g. Ang IV) and residues lacking the Phe^8 side chain (e.g. [Sar^1, Ile^8]-Ang II) but not for peptides such as Ang II (1–7) which lacks the carboxy terminus eighth residue [16]. Interaction (c) is the induction interaction between the receptor and the side chain of Phe^8 of Ang II and related agonists, which is believed to involve His^{256} of the receptor [18]. Both Ang IV and [Sar^1]-Ang II can form this interaction and hence both are agonists at the wild type receptor. However, since [Sar^1, Ile^8]-Ang II does not possess the Phe^8 side chain, it is an antagonist. Interestingly, all three ligands can activate the N111G CAM AT$_1$ receptor, suggesting that the ligand–receptor Phe^8-His^{256} interaction is not required for peptide interactions with the R* state. In addition to the side chain of Phe^8, the aromatic side chain of Tyr^4 has previously been implicated in the induction event [10]. However, in their study [Sar^1, Ile^4]-Ang II displayed reasonable efficacy (approx. 70%), with a marked increase in EC$_{50}$ relative to [Sar^1]-Ang II at the wild type receptor (approx. 10-fold). This suggests that the aromatic side chain of Tyr^4 is more likely to be involved in interaction (b), rather than induction (c). Interaction (d) is a high affinity interaction between the peptides and the active state of the receptor which also promotes G protein coupling. Combining past [10] and present data it would appear that this interaction does not require residues 1 and 2 or the aromatic side chains of Tyr^4 and Phe^8 of Ang II. All the peptides used in our study can form this interaction and we therefore cannot dissect any further detail.

Previously, multiple conformational states of the AT$_1$ receptor have been reported based on the differential ability of Ang II and [Sar^1, Ile^4, Ile^8]-Ang II to induce inositol phosphate signalling, receptor internalization and receptor phosphorylation of wild type and N111G CAM AT$_1$ receptors [19]. Of particular interest were the observations that the N111G CAM AT$_1$ receptor did not undergo agonist-induced phosphorylation, whilst phosphorylation of the wild type AT$_1$ receptor could be induced by [Sar^1, Ile^4, Ile^8]-Ang II, despite this ligand's inability to stimulate inositol phosphate signalling. These data suggest that distinct ligand–receptor conformations may exist, one a non-signalling conformation which surprisingly is a substrate for phosphorylation and a second

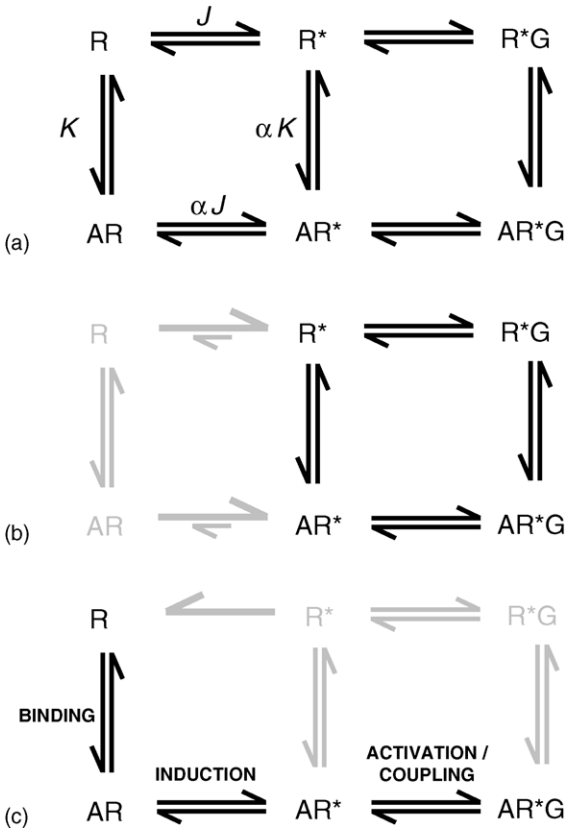

Fig. 4 – The eTCM and models derived from the eTCM explaining the activation of the wild type and N111G CAM AT_1 receptors. The activation of the wild type and N111G CAM AT_1 receptors are shown within the context of two radically different reaction schemes (b and c) which are both sub-sets of the eTCM (a). (a) Represents the eTCM. (b) This model represents the TCM and can be used to account for the properties of the N111G CAM AT_1 receptor. (c) This model represents an induction scheme which can be envisaged as a subset of the eTCM in the situation where R^* cannot form in the absence of agonist. This classic induction-based scheme requires three events: (1) ligand binding; (2) induction of the active conformation of the receptor; (3) coupling of G protein and consequent activation of the signalling cascade and can be used to account for the properties of the wild type AT_1 receptor.

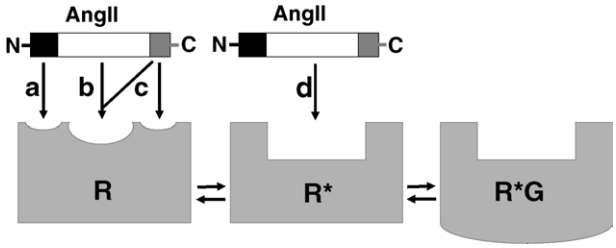

Fig. 5 – Schematic of human AT_1 receptor activation by angiotensin II. The different conformational states of the three forms of the receptor R, R^* and R^*G are represented schematically above. Binding to the R form of the receptor is represented by the wild type receptor while the R^* form is represented by the N111G CAM AT_1 receptor. Four interactions between the peptide ligands and the receptor (a–d) are envisaged. (a) An affinity-enhancing component of the docking interaction involving Arg^2 of ligand and Asp^{281} of receptor. (b) Interaction between receptor and residues 3–7, as well as the main chain of residue 8, of Ang II and related peptides. (c) Induction interaction between receptor and Phe^8 of Ang II and related agonists. (d) High affinity interaction between residues 3–7, as well as the main chain of residue 8, of Ang II and related peptides, which also promotes G protein coupling.

active conformation which couples to the G protein. Within the context of our model, these conformations may be represented by AR and AR^*G, respectively (Fig. 4c). The non-signalling conformation is predicted to be bypassed in the N111G CAM AT_1 receptor (Fig. 4b), which is in keeping with the observed lack of agonist-induced phosphorylation [19].

In summary, we have defined and clarified the principal interaction sites within the peptide ligands which are critical for both binding and induction of the active conformation of the AT_1 receptor. We have demonstrated that Arg^2 is not involved in inducing the formation of the active state R^*, but rather it is critical only for high affinity binding to the inactive R state. On the other hand, the Phe^8 side chain is critical for the induction of R^*, but not for binding affinity or for the coupling to G-protein phase. The resultant ligand binding and receptor activation model thus clarifies the properties of several peptide ligands observed at both the wild type and N111G CAM AT_1 receptors. Finally, we have used a modification of the eTCM to define a model for peptide binding and activation at both the wild type and N111G CAM AT_1 receptors. Despite the inclusion of conformational induction within the model, there is no requirement for defining an additional ''pre-activated'' receptor conformation R′. Rather, the data can be interpreted by envisaging that the wild type AT_1 receptor operates via a subset of the eTCM equilibrium scheme, such that its activation is dependent upon an induction stage, while the N111G CAM AT_1 receptor operates via a different subset of the scheme in which the induction stage is bypassed. The difference in the routes through the reaction scheme is solely due to the ability of the N111G CAM (but not wild type) AT_1 receptors to spontaneously form the R^* state in the absence of agonist.

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