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When 6 is 9: 'Uncoupled' AT_1 receptors turn signalling on its head

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Abstract. The type 1 angiotensin receptor (AT_1) activates an array of intracellular signalling pathways that control cell and tissue responses to the peptide hormone angiotensin II (AngII). The capacity of AT_1 receptors to initiate and maintain such signals has typically been explained on the basis of conventional heterotrimeric guanine nucleotide binding protein (G protein) activation, specifically $G_{q/11}$. Accumulating evidence from studies utilising a variety of AT_1 receptor mutants and AngII analogues indicates that some important downstream effects of AT_1 receptors are independent of classical G protein coupling. Importantly, AT_1 receptor-mediated endocytosis, tyrosine phosphorylation signalling and mitogen-activated protein kinase activation as well as transactivation of the epidermal growth factor receptor can occur in $G_{q/11}$-uncoupled receptor mutants. These observations point to a functional partitioning of AT_1 receptor signals that permits separation of short-term AngII actions (e. g., vasoconstriction) from more extended events, such as pathological cell growth in heart and blood vessels, and may open up new avenues for selective antagonism.

Key words. Angiotensin; type 1 angiotensin receptor; AT_1; G protein-coupled receptors; intracellular signalling; EGF receptor transactivation; MAPK.

Introduction

The type 1 angiotensin receptor (AT_1) is a 359-amino acid, seven transmembrane-spanning (7TM) receptor (see fig. 1) that mediates most of the important biological actions of angiotensin II (AngII). AngII-stimulated AT_1 receptors strongly activate heterotrimeric guanine nucleotide binding proteins (G proteins), most notably $G_{q/11}$, which couple to phospholipase C-β (PLC), leading to the hydrolysis of membrane phospholipids and the generation of diacylglycerol and inositol trisphosphate (IP_3). Diacylglycerol and IP_3 stimulate protein kinase C (PKC) and mobilise intracellular calcium, respectively, and these effectors are thought to mediate most of the well-established acute responses to AngII, including vasoconstriction, aldosterone biosynthesis and thirst/salt appetite. AngII activation of AT_1 receptors also stimulates the activity of soluble and receptor tyrosine kinases and strongly promotes mitogen-activated protein kinases (MAPKs). These latter signals appear to contribute to the known growth-promoting actions of AngII on cardiac, vascular and renal cells, yet the molecular mechanisms that link activated AT_1 receptors to tyrosine kinase pathways are not fully understood. Accumulating evidence indicates that AT_1 receptors can cross-talk to other receptor systems, principally via the transactivation of epidermal growth factor receptors, and this is closely aligned with AngII-mediated cell growth [1, 2]. For a comprehensive examination of AT_1 receptor signalling, the reader is referred to an excellent review [3] and other articles in the present series [N. J. Smith, et al.].

Concurrent with the activation of signalling through the AT_1 receptor, signalling-related kinases, such as PKC and specific G protein-coupled receptor (GPCR) kinases (GRKs) are activated, which phosphorylate primarily serine residues within the carboxyl-terminus of the receptor. GRK-dependent phosphorylation within the central region of the carboxyl-terminus targets the receptor for interaction with regulatory proteins termed β-arrestins, which prevents further association with G proteins and leads to

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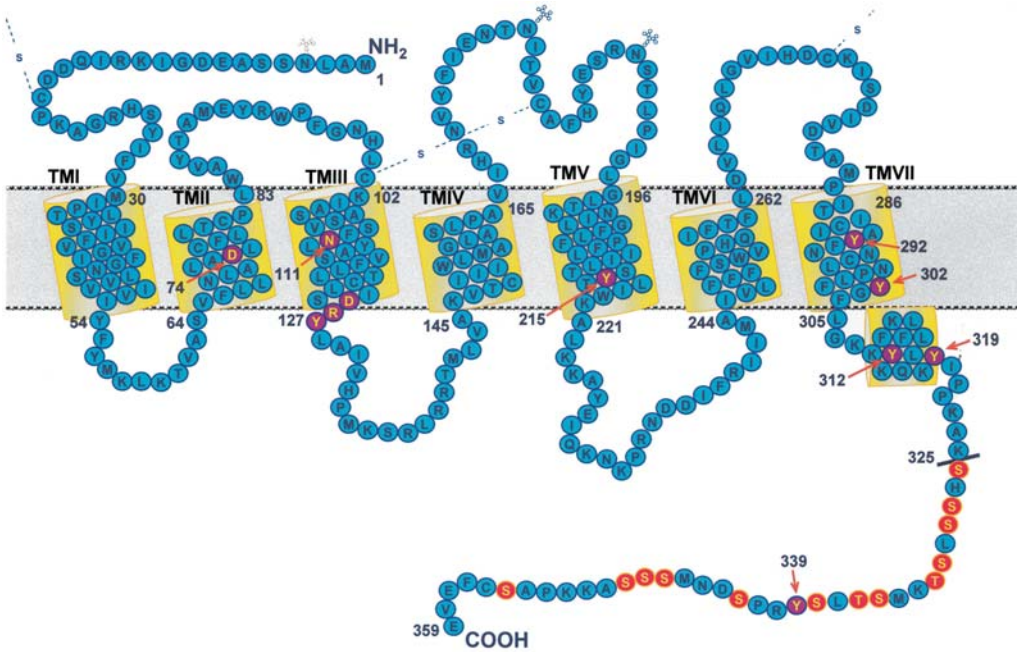

Figure 1. Schematic representation of the rat AT_1 receptor. The 359-amino acid AT_{1A} receptor is a 7TM protein (transmembrane helices I–VII are identified); the position of key residues discussed in this review and relevant to $G_{q/11}$-PLC uncoupling are highlighted (yellow lettering on purple). Carboxyl-terminal serines and threonines (potential phosphorylation sites) are also highlighted (yellow lettering on red).

desensitisation of signalling. β-Arrestins also interact with components of the internalisation machinery (e.g., clathrin, AP2 and *N*-ethylmaleimide-sensitive protein) and guide activated/phosphorylated receptors into clathrin coated pits for receptor endocytosis. Based on the classification of Oakley et al. [4], AT_1 receptors are class B GPCRs in that they bind and traffic both ubiquitous forms of β-arrestin (β-arrestin 1 and 2) to deep core, endocytic vesicles [4]. In addition, β-arrestins act as scaffolds to recruit additional signalling (e.g., the tyrosine kinase, c-Src, and members and upstream regulators of the MAPK family) and regulatory molecules (e.g., the ubiquitin ligase, Mdm2, phosphodiesterase, and the cytoskeletal protein, Ral-GDS) that further impinge upon receptor activity and may allow secondary phases of receptor signalling during the process of internalisation and targeting to endocytic vesicles. The role of arrestins in GPCR and AT_1 receptor function has been recently reviewed [5–9].

Clearly, the capacity of AT_1 receptors to activate the $G_{q/11}$-PLC signalling pathway, to transactivate growth factor receptors, to be phosphorylated, bound by arrestins and internalised adds complexity and flexibility to AT_1 signalling. Traditionally, $G_{q/11}$-PLC signalling has been used to explain most AngII-mediated responses, but this view is too narrow and requires expansion. Specifically, it is now believed that 7TM receptors transit through multiple, functionally separate conformational states during the complex process of receptor activation and deactivation [10]. In this review, we will focus on recent data utilising 'uncoupled' mutants of the AT_1 receptor and the insights they give us into the processes that control receptor signalling and regulation.

Early clues

Almost a decade before the idea of multiple functional receptor conformations/states, variously termed ligand-directed trafficking of receptor signals or biased agonism, moved from obloquy to mainstream [10], research into the activity of AT_1 receptors revealed early indications of such phenomena. Most notably, Hunyady et al. [11] demonstrated that AT_1 receptors bearing mutations in the conserved aspartic acid (Asp^{74}) (see fig. 1) in TM2 displayed significantly inhibited G protein coupling and IP_3 signalling, yet had almost unaffected AngII-stimulated internalisation. Moreover, another receptor mutant was constructed with a deletion of residues 221–226 in the third intracellular loop, in combination with an $Asp^{74}Asn$ substitution that showed abrogated G protein coupling (indicated by no effect of GTPγS on [^{125}I]AngII binding in membrane derived from cells expressing these receptors) and no IP_3 response to AngII stimulation. Despite uncoupling for G protein and classical signalling, this mutated AT_1 receptor retained a strong capacity to internalise, providing compelling evidence that the structural requirements for receptor endocytosis differ from those necessary for productive coupling to $G_{q/11}$-PLC. This seminal observation was soon supported by evidence that

substituted peptide 'antagonists' of AngII, specifically Sar^1Ile^8-AngII, which poorly activate IP_3 production via wild-type AT_1 receptors, robustly stimulate AT_1 receptor internalisation to levels equivalent to the parental peptide [12, 13]. More recent studies [14, 15] using green fluorescent protein (GFP)-tagged AT_1 receptors and confocal microscopy have confirmed that non-signalling AngII analogues can indeed internalise and traffic the AT_1 receptor, including an interesting analogue, $Sar^1Ile^4Ile^8$-AngII, that unlike Sar^1Ile^8-AngII (which is a weak agonist) generates no measurable inositol phosphate accumulation [16]. Remarkably, $Sar^1Ile^4Ile^8$-AngII also promotes strong phosphorylation of the AT_1 receptor [17, 15] (which most likely explains its capacity to cause receptor internalisation) as well as stimulating MAPK activity, albeit at a reduced level [15, 18].

Apart from convincingly supporting the idea of separate, functional conformational states for the AT_1 receptor, these data also strongly argue against a linear model of receptor activation from ligand binding to G protein activation, receptor phosphorylation, arrestin binding, internalisation and desensitisation. More likely, receptor transition is far more complex, with separate signals and activities reflecting unique receptor states and discrete temporal and spatial arrangements for activation and deactivation.

Constitutively active AT_1 receptors

Consistent with the idea that receptors spontaneously isomerise between inactive and active conformational states, specific mutations in the AT_1 receptor lead to constitutive (or ligand independent) activity. The first described mutation of this type for the AT_1 receptor was at Asn^{111} in TM3 [16, 19], although saturation mutagenesis of the AT_1 receptor [20] as well as others [21] reveals that all regions of GPCRs potentially contribute to receptor activation, including the N- and C-termini. Substitutions of Asn^{111} to smaller amino acids like alanine and glycine revealed strong constitutive coupling to $G_{q/11}$-PLC as measured by high affinity binding of agonists and dramatically increased basal inositol phosphate production [22]. Interestingly, a recent study [23] indicates that despite this constitutive activity, the $[N^{111}G]AT_1$ receptor is paradoxically uncoupled from $G_{q/11}$. Thus, constitutive activity probably reflects an intermediate conformational state of the receptor that has a higher capacity for catalytic activation of the G proteins without promoting stable receptor-G protein interactions.

The unique conformational state of the $[N^{111}G/A]AT_1$ mutants is supported by the observation that analogues like $Sar^1Ile^4Ile^8$-AngII [16] and AngII3-8 (AIV) [24], which are inactive at the wild-type receptor, can fully activate $G_{q/11}$-PLC signalling via the constitutively active receptor. Moreover, we reported that despite their constitutive activity at $G_{q/11}$-PLC, the $[N^{111}G]AT_1$ and $[N^{111}A]$ AT_1 receptors are very poorly phosphorylated, both basally and following AngII stimulation. This was somewhat surprising because these mutant receptors displayed near wild type rates of receptor internalisation following AngII stimulation. Most recently, Miura and Karnik [25, 26] and Boucard et al. [27] used the substituted cysteine accessibility method and MTSEA sensitivity to provide experimental evidence for conformational differences between the wild-type and constitutively active AT_1 receptors. Their data point to structural changes during receptor activation that involve rotation of TM2, its interaction with TM7 and exposure of amino acids in TM7 to a more aqueous environment.

$G_{q/11}$ 'uncoupled' AT_1 receptor mutants

Many studies have reported mutations of the AT_1 receptor that result in varying degrees of uncoupling from $G_{q/11}$-PLC (see examples below and fig. 1). Some caution should be applied when interpreting the degree of uncoupling reported, because some of these mutant receptors express poorly and the level of inositol phosphate signalling requires normalisation for receptor expression at the cell surface. In addition, [^{125}I]-AngII binding of some supposedly uncoupled receptor mutants retains significant sensitivity to the non-hydrolysable GTP analogue, GTPγS, indicating reasonable G protein coupling.

G protein-independent signalling

In 2001, Doan et al. [28] introduced an AT_1 receptor mutant with interesting features. The mutant, termed M5, contained phenylalanine substitutions of five tyrosines (Tyr^{292}, Tyr^{302}, Tyr^{312}, Tyr^{319}, Tyr^{339}) located in the carboxyl-terminal region of the receptor. Presumably, the rationale for these substitutions was to prevent putative tyrosine phosphorylation of the receptor that had been previously linked to growth factor-type signalling via the AT_1 receptor. When the M5 mutant was stably expressed in Chinese hamster ovary cells (CHO-K1 cells), stimulation with maximal concentrations of AngII failed to activate IP_3 signalling or generate calcium transients, indicating a loss of functional G_q coupling. In contrast, AngII stimulation of the M5 receptor induced a strong increase in the tyrosine phosphorylation of the soluble tyrosine kinase Jak2 and its downstream effector, the transcription factor STAT1, to levels comparable to the wild-type AT_1 receptor. Thus, the M5 receptor is capable of coupling to tyrosine kinase signalling in the absence of G_q activation. This conclusion was confirmed using another G_q-uncoupled AT_1 receptor ($[D74E]AT_1$), which also activated the Jak-STAT pathway, and by the retention of Jak-STAT activation in response to AngII in a fibroblast cell line lacking $G_{q/11}$. Despite much evidence that $G_{q/11}$ activation is an

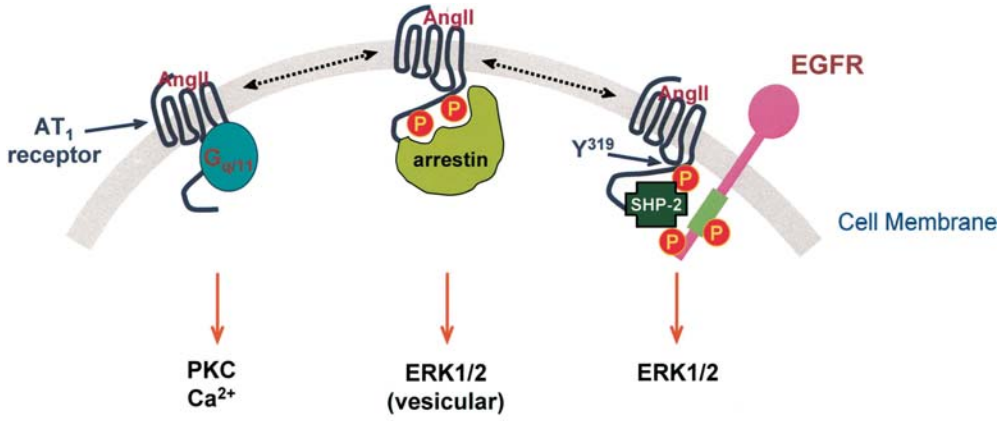

Figure 2. Separation of signalling via AT_1 receptors. Three main mediators of AT_1 receptor activation are shown. The classical coupling to $G_{q/11}$ results in PKC activation and intracellular calcium mobilisation. Binding of phosphorylated receptors by arrestins can terminate initial $G_{q/11}$ signals and promotes receptor endocytosis during which β-arrestin2 acts as a scaffold to sequester ERK1/2 into AT_1-containing endocytic vesicles. AT_1 receptors can also transactivate EGF receptors to activate ERK1/2 via a variety of mechanisms (intracellular soluble tyrosine kinases, EGF-like ligand shedding form the cell surface or via direct interaction, as suggested by Seta and Sadoshima [35]).

important mediator of cell growth through 7TM receptors [29], the M5 receptor stimulated cell proliferation in an AngII- and tyrosine kinase-dependent manner, indicating that the capacity to activate tyrosine phosphorylation signalling may be more important than $G_{q/11}$ in promoting growth.

The activation of tyrosine kinase signalling in the absence of G protein coupling is not confined to the M5 and $[D^{74}E]AT_1$ mutants. Mutation within the highly conserved DRY ($Asp^{125}Arg^{126}Tyr^{127}$) motif, located at the junction of TM3 and intracellular loop 2, also produces a receptor that is uncoupled from heterotrimeric G proteins [30, 31]. Seta et al. [32] reported that expression of an $[D^{125}G, R^{126}G, Y^{127}A, M^{134}A]AT_1$ mutant (referred to as AT1a-i2m) in CHO-K1 cells produced a receptor that was uncoupled from G proteins, as indicated by GTPγS-resistant radioligand binding on membranes prepared from these cells. In intact cells expressing the AT1a-i2m mutant, AngII failed to generate inositol phosphates or inhibit forskolin-mediated cyclic AMP (cAMP) accumulation, confirming uncoupling from $G_{q/11}$ and G_i, respectively. In contrast, the AT1a-i2m receptor rapidly and potently activated the soluble tyrosine kinase, Src, in a manner indistinguishable from the wild-type AT_1 receptor. Src activation by AT1a-i2m was not inhibited by a specific PKC inhibitor, by chelating intracellular calcium or by overexpression of a member of the regulator of G protein signalling family (RGS4), further verifying its independence from $G_{q/11}$-PLC signalling. Moreover, a carboxyl-terminus-truncated version of the AT_1 receptor ($[1\text{-}309]AT_1$), which is capable of fully activating inositol phosphate generation, did not display AngII-mediated Src activation. These data strongly suggest that the carboxyl-terminus distal to 309, but not $G_{q/11}$ coupling, controls Src activation.

In addition to Src activation, the AT1a-i2m receptor also coupled to the extracellular signal regulated kinase (ERK) MAPKs, as did the wild-type receptor. ERK1/2 activation by the wild-type receptor was partially blocked by the Src inhibitor PP1, whereas ERK activation by AT1a-i2m was completely inhibited. AT1a-i2m-mediated ERK activation was also abrogated by co-expression of a dominant-negative version of Ras, the small GTP-binding protein commonly upstream of ERK signalling. Interestingly, the coupling of the wild-type AT_1 receptor to ERK was weakly affected by the Ras mutant, but was strongly inhibited in the combined presence of a PKC inhibitor and dominant-negative Ras. These observations suggest that in the absence of a classical $G_{q/11}$-PLC signal (as would occur for a G protein-uncoupled receptor mutant, like AT1a-i2m), ERK activation is solely dependent upon a Ras-Src pathway. Under such conditions, activated ERKs fail to translocate to the nucleus and phosphorylate targets, such as the nuclear transcription factor Elk1. A major difference between this study and that of Doan et al. [28] is that the AT1a-i2m receptor mutant failed to stimulate cell proliferation, whereas the similarly uncoupled M5 mutant strongly promoted cell growth. The reasons for this are unclear, as both M5 and AT1a-i2m appear to possess the same phenotype – uncoupled from $G_{q/11}$ but strongly linked to tyrosine kinase pathways.

More recently, alanine-scanning mutational analysis of the conserved DRY motif with respect to receptor activation has been reported by Gaborik et al. [33]. While single substitution of Asp^{125} ($[D125A]AT_1$) or Arg^{126} ($[R126A]AT_1$) only partially inhibited receptor activation, combined mutation (termed $[D^{125}R^{126}Y^{127}/AAY]AT_1$) completely abrogated inositol phosphate signalling. Mutation of adjacent residues in the second intracellular loop (specifically, Ile^{130} and Met^{134}) also

showed impaired $G_{q/11}$/PLC coupling. Significantly, for the [DRY/AAY]AT_1 and [I^{130}A]AT_1 and [M^{134}A]AT_1 mutants, receptor internalisation was only modestly reduced, and this supports a separation of receptor endocytosis from receptor signalling for the AT_1 receptor. Using an indirect measure of ERK activation (Elk1 activation), these authors demonstrated that a reasonable correlation exists between the level of nuclear ERK activity and $G_{q/11}$-PLC responses, confirming the data of Seta et al. [32].

AT_1 receptor transactivation of EGFR

It is now accepted that many 7TM receptors can promote tyrosine phosphorylation and transactivation of growth factor receptors, such as the EGFR, and that this can play an important role in MAPK activation and cell growth [2, 34]. It is not clear whether this process involves or requires G protein activation. The mechanism can involve either Ca^{2+}-dependent activation of cytoplasmic kinases, such as Src and Pyk2, or a metalloprotease-induced shedding of EGF-like ligands from the cell surface. The ability of AT_1 receptors to promote transactivation of the EGFR has been demonstrated in a variety of cell systems, including liver, vascular smooth muscle, renal, prostate and heart. In trying to understand how $G_{q/11}$ uncoupled AT_1 receptors can marshal tyrosine kinase and MAPK activation, one strong possibility is via EGFR transactivation. Future studies should address directly whether the various G-protein-uncoupled AT_1 receptor mutants discussed above retain the capacity to transactivate EGFRs.

One interesting proposal [35] is that EGFR transactivation may involve an interaction between the AT_1 receptor carboxyl-terminus and the EGFR, supporting previous studies that predict functional complexes between 7TM receptors and tyrosine kinase receptors [36]. In COS-7 cells, co-transfected with the EGFR and wild-type AT_1 receptor, AngII stimulation led to a rapid (maximal at 5 min) tyrosine phosphorylation of the EGFR, indicating that the AT_1 receptor causes transactivation of the EGFR in this model system. To examine the contribution of the AT_1 receptor carboxyl-terminus to AngII-mediated EGFR transactivation, two carboxyl-terminally truncated versions ([1-338]AT_1 and [1-311]AT_1) of the AT_1 receptor were separately co-expressed with EGFR, and their capacity to tyrosine-phosphorylate the EGFR following AngII stimulation was determined. Activation of [1-338]AT_1 led to EGFR phosphorylation, whereas [1-311]AT_1 did not, suggesting that a determinant in the region 311–338 was involved. Within this region is the highly conserved YIPP motif (residues 319–322), which others have reported binds a number of signalling molecules (PLCγ, JAK, SHP-2) [37]. Single-point mutation of Tyr^{319} to phenylalanine completely inhibited AT_1 receptor-dependent EGFR phosphorylation, suggesting that phosphorylation at Tyr^{319} in the wild-type receptor may be involved. Consistent with that idea, substitution of Tyr^{319} with a phosphate-mimicking amino acid (glutamic acid) enhanced basal EGFR transactivation. To confirm that Tyr^{319} was indeed phosphorylated in the activated AT_1 receptor, Seta and Sadoshima [35] raised polyclonal antibodies to a phosphopeptide spanning Tyr^{319} (QLLK [pY^{319}]IPPKAKS). Using this antibody to both immunoprecipitate and detect the activated AT_1 receptor, they reported that the wild-type receptor, but not [Y^{319}F]AT1, was tyrosine phosphorylated in a transient manner in response to AngII stimulation. Overexpression of a minigene encoding the entire AT_1 carboxyl-terminus (residues 292–359), but not the same sequence encompassing a Y319F mutation, inhibited AngII-dependent EGFR phosphorylation. Taken together, these data suggest that activation of the AT_1 receptor leads to tyrosine phosphorylation of Tyr^{319} and that this plays an important role in mediating EGFR transaction.

The exact role that phosphorylation of Tyr^{319} plays in EGFR transactivation remains unknown, but remarkably Seta and Sadoshima [35] went on to demonstrate that the activated AT_1 receptor and the EGFR co-immunoprecipitate and that this association was lost for [Y319F]AT_1. The interaction was inhibited by co-expression of the tyrosine phosphatase, SHP-2, which has been shown to associate with the AT_1 receptor via the YIPP motif. Whether this was due to inhibition of the SHP-2 phosphatase activity or via competition for the phosphorylated Tyr^{319} binding site is not clear. The interpretation favoured by the authors is that SHP-2 acts as a scaffold to mediate the apposition of the AT_1 receptor and EGFR. Interestingly, [Y319F]AT_1 retains a capacity to generate inositol phosphates, activate Src, initiate the Jak-STAT pathway and stimulate ERK MAPK. In contrast to the wild-type receptor, where AngII-induced ERK1/2 activation was completely dependent on the EGFR, ERK activation of [Y319F]AT_1 was insensitive to EGFR inhibition with AG1478. Instead, [Y319F]AT_1-mediated ERK signalling was dependent upon Src and Ras. Finally, although [Y319F]AT_1 was able to robustly couple to inositol phosphates, Src, Ras, Jak-STAT and ERK, this receptor did not promote proliferation in cardiac fibroblasts, indicating a central role for the EGFR, but not ERK specifically, in the growth response to AT_1 activation.

β-arrestins as mediators of AT_1 signalling

In addition to desensitising and internalising 7TM receptors, β-arrestins also act as scaffolds for signalling molecules, including those that lead to activation of MAPKs [5–8, 38]. β-arrestins bind the phosphorylated carboxyl-terminus of the AT_1 receptor and mediate receptor endocytosis; they also recruit components of the ERK MAPK cascade to the activated, internalising receptor, resulting

in the targeting of activated ERK into cytoplasmic endocytic vesicles. This sequestered ERK is excluded from the nucleus and is not transcriptionally active. The functional consequence of potentially different pools of AT_1-mediated ERK is not fully appreciated, nor is the relative contribution of traditional G protein coupling, EGFR transactivation and/or β-arrestin scaffolding to total ERK activation.

In a very recent study, Wei et al. [18] investigated the link between $G_{q/11}$ activation and β-arrestin for ERK signalling by the AT_1 receptor. AngII activation of the $G_{q/11}$-uncoupled [DRY/AAY]AT_1 mutant described above [33] or stimulation of the wild-type AT_1 receptor with the $G_{q/11}$-PLC inactive AngII analogue, $Sar^1Ile^4Ile^8$-AngII, predicably did not generate inositol phosphates in HEK-293 cells. In contrast, both these paradigms yielded significant ERK1/2 activation, which were ~50% that observed for AngII stimulation of the wild-type AT_1 receptor. We previously reported that $Sar^1Ile^4Ile^8$-AngII could activate ERK1/2 via expressed AT_1 receptors in CHO-K1 cells [15]. In addition, Wei et al. [18] showed that β-arrestin2-GFP translocated into endocytic vesicles following AngII activation of [DRY/AAY]AT_1 and $Sar^1Ile^4Ile^8$-AngII stimulation of the wild-type receptor. To examine the contribution of β-arrestin2 to ERK1/2 activation, small interfering RNA (siRNA) was used to knock down the endogenous expression of β-arrestin2. For the wild-type receptor stimulated with AngII, β-arrestin2 knockdown reduced maximal ERK1/2 activation by ~50% – this residual component was inhibited by Ro-31-8425, a PKC inhibitor. Reducing β-arrestin2 levels totally prevented ERK1/2 activation by $Sar^1Ile^4Ile^8$-AngII on wild-type receptors and by AngII on [DRY/AAY]AT_1 receptors, whereas PKC inhibition had no effect. These data suggest that AT_1-mediated ERK activation comprises two separate components – one involves $G_{q/11}$ activation of PKC and the other is independent of PKC and requires β-arrestin2. They also provide strong support for the idea that 7TM receptors can attain separate, functional conformations that show clear preference for specific receptor activities. Given that β-arrestin2-mediated ERK1/2 activation is not translocated to the nucleus but is retained in endocytic vesicles in the cytoplasm [32], it should follow that the ERK signal generated by $Sar^1Ile^4Ile^8$-AngII and [DRY/AAY]AT_1 receptors may not be sufficient to promote a growth response. While this needs to be tested experimentally, it does raise the question: if not ERK, what are the bone fide signals that underlie AT_1-mediated cell growth, especially that observed when $G_{q/11}$ is uncoupled?

The future

Future studies need to ascribe precise biological outcomes to cell- and tissue-specific AT_1 receptor signals. The receptor mutants and analogues thus far described may be useful in this endeavour. Also, the components of AT_1 receptor activation that come from a strictly $G_{q/11}$ response versus EGFR transactivation or β-arrestin need further scrutiny. Cells lines (mouse embryonic fibroblasts) derived from non-viable knockout mice for $G_{q/11}$ or β-arrestin1/2 will be increasingly used to provide null backgrounds for reconstitution of specific receptor outcomes. A full description of the mechanism(s) of non-$G_{q/11}$ signalling is paramount. The data of Seta and Sadoshima [35] detailing tyrosine phosphorylation of Tyr^{319} in the AT_1 receptor and its relevance to EGFR transactivation are provocative and so important that they need to be independently confirmed. Although others have also reported tyrosine phosphorylation of the activated AT_1 receptor, it is not generally accepted. It is our experience that the AT_1 receptor is robustly phosphorylated following AngII stimulation, but not on tyrosine (as detected by blotting of activated, immunoprecipitated AT_1 receptors with anti-phosphotyrosine antibodies). This does not preclude some weak tyrosine-based phosphorylation, but it seems astonishing that such a potentially functionally important event is not readily demonstrable. Other plausible mechanisms for 7TM receptor transactivation of EGFRs, such as HB-EGF shedding or soluble tyrosine kinase activation, should also be closely examined.

Finally, we clearly need to know more about the precise activity states of AT_1 receptors. Additional signalling-specific receptor mutants and analogues will undoubtedly be uncovered. These, and the current armoury of analogues and constitutive or signalling-specific AT_1 receptor mutants, should prove very useful in modelling such transitional states of the receptor based on the high-resolution structure of rhodopsin [39]. As the purification and crystallisation of membrane proteins improves, perhaps some of the mutant receptors, in complex with specific AngII analogues, will be purified and crystallised to provide important structural information on the separate receptor states. In the meantime, alternative approaches at probing the structure-function aspects of 7TM receptors and their putative receptor states, such those recently elaborated [40, 41], need to be applied to the AT_1 receptor.

The functional compartmentalisation of signals emanating from the AT_1 receptor, revealed using $G_{q/11}$ uncoupled receptors and analogues of AngII, has important biological and therapeutic implications. The first is that AngII-induced signals can be temporally and physically separated, which makes sense given the diverse nature of responses to this peptide hormone (for example, vasoconstriction versus cell growth). Logically, short-term constriction of a blood vessel should not commit the contracting vascular smooth cells to inappropriate proliferation. Next, it is probable that the specific cellular environment and its complement of GRKs, arrestins and

other regulatory partners favour various functional receptor states or signalling compartmentalisation and thereby the strength and duration of any specific signal. Lastly, as is being increasingly appreciated [10, 18], the unique features possessed by analogues, such as $Sar^1Ile^4Ile^8$-AngII, means it should be possible to screen for non-peptide analogues of AngII, as well as ligands for other 7TM receptors, that can selectively activate or antagonise individual receptor activities. Such development may produce drugs that selectively control distinct aspects of receptor function – perhaps effectively separating clinical control of blood pressure from pathological growth of cardiomyocytes or vascular smooth muscle cells.

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