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Type 1 angiotensin receptor pharmacology: Signaling beyond G proteins

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Abstract

Drugs that inhibit the production of angiotensin II (AngII) or its access to the type 1 angiotensin receptor (AT_1R) are prescribed to alleviate high blood pressure and its cardiovascular complications. Accordingly, much research has focused on the molecular pharmacology of AT_1R activation and signaling. An emerging theme is that the AT_1R generates G protein dependent as well as independent signals and that these transduction systems separately contribute to AT_1R biology in health and disease. Regulatory molecules termed arrestins are central to this process as is the capacity of AT_1R to crosstalk with other receptor systems, such as the widely studied transactivation of growth factor receptors. AT_1R function can also be modulated by polymorphisms in the *AGTR* gene, which may significantly alter receptor expression and function; a capacity of the receptor to dimerize/oligomerize with altered pharmacology; and by the cellular environment in which the receptor resides. Together, these aspects of the AT_1R "flavour" the response to angiotensin; they may also contribute to disease, determine the efficacy of current drugs and offer a unique opportunity to develop new therapeutics that antagonize only selective facets of AT_1R function.

Keywords: Angiotensin; Type 1 angiotensin receptor; Arrestin; Dimerization; Polymorphism; G protein

Abbreviations: ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AngI, angiotensin I; AngII, angiotensin II; AT_1R, type 1 angiotensin receptor; AT_2R, type 2 angiotensin receptor; BRET, bioluminescence resonance energy transfer; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; RAS, renin–angiotensin system; RNAi, RNA interference; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; TM, transmembrane helix.

Contents

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1. Introduction

1.1. The renin–angiotensin system

The renin–angiotensin system (RAS) is one of the most comprehensively researched and clinically relevant homeostatic systems in human physiology. An ever-expanding, and almost insurmountable,[1] body of literature exists pertaining to the workings of this system and its contribution to cardiovascular, neural, endocrine and metabolic biology and disease. Appropriately, a number of comprehensive reviews exist that summarize the vast array of experimental and clinical research into the renin–angiotensin, its components and their regulation, and the relevance of this system to human health and dysfunction (de Gasparo et al., 2000; Dzau et al., 2002; Unger, 2002; Zaman et al., 2002; Berk, 2003; Lavoie & Sigmund, 2003; Azizi & Menard, 2004; Re, 2004; Casas et al., 2005; Sakai & Sigmund, 2005; Bernstein, 2006; Fleming et al., 2006). It is not our intention to recapitulate much of this generalist information; instead, where appropriate, we direct the reader to current and accessible reviews. Here, we will provide a brief overview of the RAS, including some recent advances in the complexity of angiotensin generation and reception.

1.1.1. The RAS: an update

The basic RAS persists - a single, obligate precursor protein, angiotensinogen, remains the source of all angiotensin peptides. Circulating angiotensinogen (from liver) is primarily cleaved by the protease, renin (from kidney), to yield a 10 amino acid peptide, angiotensin I (AngI), which in turn is processed by angiotensin converting enzyme (ACE) (in lung) to produce the active octapeptide, angiotensin II (AngII). AngII binds with high affinity to 2 distinct receptors, the type 1 and type 2 angiotensin receptors (AT_1R and AT_2R)—both are 7 transmembrane-spanning receptors that belong to the G protein-coupled receptor (GPCR) superfamily. The important cardiovascular actions of AngII, including regulation of arterial blood pressure and water-salt balance, are predominantly mediated by the AT_1R in target tissues such as the blood vessels, kidney, brain and heart. The role of the AT_2R is less clear (Steckelings et al., 2005)- its G protein coupling and signal transduction mechanisms remain poorly defined and its function as an antagonist of the AT_1R is not universally accepted.

The complexity of this elementary RAS continues to evolve (Kurdi et al., 2005; Hunyady & Catt, 2006) (see Fig. 1). First, the generation of angiotensin within the circulation is complemented by local tissue RASs in a number of tissues, including the brain, kidney, heart, and blood vessels (Re, 2004). Angiotensin generated locally has important homeostatic functions and likely contributes to disease, yet the exact mechanism by which angiotensin is produced, in areas such as the brain, remains a source of controversy and extensive research. Second, polymorphic variations of the various RAS components (angiotensinogen, renin, ACE, AT_1R and AT_2R) exist and these may determine the level of angiotensin production and action (Luft, 2002; Bleumink et al., 2004; Miller & Scholey, 2004; Baudin, 2005). For example, polymorphisms in angiotensinogen (most notably $M^{235}T$) may increase renin cleavage and angiotensin production and has been associated with a variety of diseases (Miller & Scholey, 2004). Similarly, genetic variations in renin (Hasimu et al., 2003) and ACE (Miller & Scholey, 2004) have been reported and associated with disease. Polymorphisms in the AT_1R gene (*AGTR1*) may also have important functional and clinical implications and these are detailed separately in Section 4.3. Third, the recent identification and cloning of angiotensin converting enzyme 2 (ACE2; Turner et al., 2004), a protein with homology (~40%) to the N-terminal catalytic domain of ACE, has revealed alternative pathways for angiotensin peptide generation. ACE2 has attracted significant interest because it

[1] More than 97,000 PubMed citations for renin/angiotensin/ACE/angiotensinogen (www.ncbi.nlm.nih.gov).

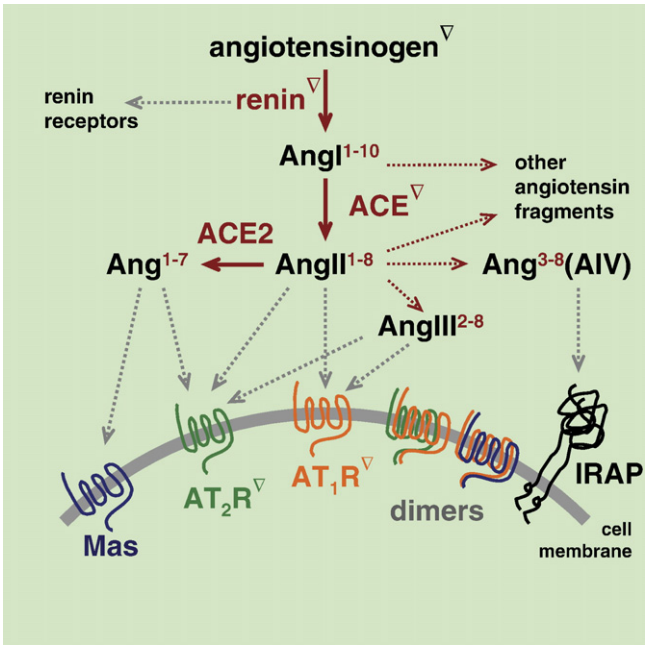

Fig. 1. An expanded RAS. The traditional processing of the precursor, angiotensinogen, by renin and ACE to yield the bioactive peptide AngII is complemented by the generation of alternative angiotensin fragments. These act through an extended family of angiotensin receptors (AT_1R, AT_2R, Mas and IRAP), which may act as monomers and/or as homo-/hetero-dimers, allowing considerable overlap and crosstalk between their respective signal transduction systems. IRAP indicates insulin regulated animo peptidase; inverted triangles indicate protein components with established polymorphisms of altered function that may associate with disease.

efficiently cleaves the carboxyl-terminal phenylalanine from AngII to generate Ang1-7, a peptide that has hypotensive actions that counteract the actions of AngII. Ablation of ACE2 in mice indicates important roles for heart and lung function related to the RAS (Crackower et al., 2002; Imai et al., 2005; Yamamoto et al., 2006). Ang1-7 reportedly binds and acts via the GPCR, Mas (Santos et al., 2003), and evidence regarding crosstalk between AT_1R and Mas is presented in Section 4.2.2.3. The generation and activity of other angiotensin peptide fragments (including Ang3-8, which appears to have its own receptor, the insulin regulated amino peptidase; Albiston et al., 2001; Albiston et al., 2003) are accepted, although the relevance to the pleiotropic actions of angiotensin remains to be fully resolved. Finally, the processing enzymes of the RAS, namely renin and ACE, may themselves be capable of signal transduction. Receptors for renin (and pro-renin) have been described, including a receptor which specifically binds the normally inactive pro-renin at the cell surface; this binding led to robust catalytic activity as well as the generation of AngII-independent intracellular signals (Nguyen, 2006). ACE is a transmembrane protein that is able to be phosphorylated within its cytoplasmic tail and can recruit signaling molecules in a manner analogous to bona fide receptors (Fleming, 2006), whereas ACE2 is also a receptor that allows internalization and infection by the SARS coronavirus (Li et al., 2003; Turner et al., 2004; Kuba et al., 2005).

1.1.2. The expanded RAS—therapeutic implications

Given the importance of angiotensin to cardiovascular homeostasis and the inherent complexity of balancing angiotensin generation and action as well as its processing into various fragments, it is not surprising that subtle, as well as overt, changes in the relative levels and activity of the precursors, processing enzymes and receptor/signaling components of the RAS might be associated with dysfunction and disease—these include, hypertension, growth and remodeling of the heart, blood vessels and the kidney, atherosclerosis, myocardial infarction, congestive heart failure, stroke and the complications of metabolic imbalance, such as diabetes. ACE inhibitors and AT_1R blockers are widely prescribed for cardiovascular disease and show efficacy; whether this results from direct effects on AngII generation and specific blocking of AT_1R signaling or indirect actions (e.g., effects on the metabolism of the vasodilator, bradykinin, during ACE inhibition or, in the case of AT_1R blockade, increased AngII "spillover" onto the AT_2R and enhanced production of AngII fragments such as Ang1-7, both of which reportedly antagonize AT_1R actions) is not completely understood. The susceptibility of certain individuals and groups to develop RAS-related diseases as well as their receptiveness (or otherwise) to therapy could reasonably reflect functional polymorphisms in the genes encoding RAS components. While many studies (with mixed success) have investigated individual mutations in angiotensinogen, renin and ACE as an explanation for RAS-associated diseases, the polygenic nature of complex diseases like hypertension means more extensive, high-throughput genotyping and haplotype association studies are required. Finally, the potential that a local RAS may contribute directly to disease opens up possibilities for selective treatment. The brain RAS is an area of particular interest in this regard as local AngII generation is closely linked to increased sympathetic outflow and hypertension. A recent study in mice used virus-based vectors to deliver small interfering RNA (siRNA) to knockdown AT_1R expression in discrete brain regions (Chen et al., 2006) and revealed region-selective modulation of AngII effects on blood pressure and water intake. This type of study as well as others aimed at modifying RAS and cardiovascular centres in the brain (Lu & Raizada, 1995; Sinnayah et al., 2004) portends application of virus-based approaches to long-term modulation of AngII actions in a defined manner.

2. Type 1 angiotensin receptor activation and signaling

2.1. Type 1 angiotensin receptor cloning and classification

AT_1R were cloned in the early 1990s (de Gasparo et al., 2000) and homology-based comparisons revealed that the AT_1R was a member of the 7-transmembrane spanning, GPCR superfamily. GPCR are the largest group of receptors in the human genome and their actions impinge on most aspects of human physiology. Not surprisingly, these receptors are major targets for drug development, and the non-peptide antagonists of the AT_1R (the "-sartans") are frontline therapeutics in the treatment of hypertension and cardiovascular disease. One

AT_1R gene (*AGTR1*) exists in humans located on chromosome 3,[2] whereas gene duplication in rodents has produced 2 highly homologous isoforms in rodents, termed AT_{1A} and AT_{1B}.

2.2. Type 1 angiotensin receptor as a G protein-coupled receptor

The AT_1R is a 359 amino acid integral membrane protein, which readily couples to heterotrimeric guanine nucleotide binding proteins (G proteins). In various cells, expressing endogenous or ectopically-introduced AT_1R, AngII stimulation leads to the activation of phospholipase C-β, hydrolysis of membrane phospholipids and the liberation of diacylglycerol (which activates protein kinase C [PKC]) and inositol trisphosphate (which mobilizes intracellular calcium), indicative of productive coupling to $G_{q/11}$. These $G_{q/11}$ signals have been linked to the classical effects of AngII on short-term vasoconstriction, aldosterone release and water and salt balance. Although most would agree that $G_{q/11}$ is the primary G protein activated by AT_1R (de Gasparo et al., 2000), evidence exists for coupling to other G protein classes, including $G_{i/o}$, $G_{12/13}$ and even G_s.

2.2.1. Receptor and signaling crosstalk

The activated AT_1R can also couple to an array of other intracellular signal transduction pathways (de Gasparo et al., 2000; Hunyady & Catt, 2006), including soluble and receptor tyrosine kinases, the mitogen-activated protein kinases (MAPK) (extracellular regulated kinases [ERK] 1/2, p38MAPK and Jun N-terminal kinase), generation of reactive oxygen species and modulation of various ion channels. The capacity to usurp tyrosine kinase-related pathways and their downstream effectors likely explains the well-established role of AngII in the growth and remodeling of the vasculature, heart and kidney that occurs during metabolic disturbances and cardiovascular disease. Multiple mechanisms have been reported that link AT_1R to these pathways including traditional $G_{q/11}$-phospholipase C pathways, the release of cytokine and growth factor ligands from cells, direct interaction between AT_1R and upstream activators and scaffolds, and the transactivation of growth factor receptors, such as the epidermal growth factor receptor (EGFR) (Smith et al., 2004; Shah & Catt, 2006; Suzuki & Eguchi, 2006). Accumulating evidence indicates that some of these signals retain an absolute requirement for G protein coupling (ostensibly $G_{q/11}$), whereas others are retained under situations where such coupling is abrogated via pharmacological inhibition, receptor mutation or ligand modification.

3. Regulation of the type 1 angiotensin receptor

3.1. GPCR phosphorylation and internalization

Like many GPCR, the AT_1R is rapidly phosphorylated following AngII stimulation by specific G protein-coupled receptor kinases (GRK) as well as second messenger-activated kinases, such as PKC (Thomas, 1999; Hunyady et al., 2000). This phosphorylation occurs primarily within the cytoplasmic tail of the receptor and the agonist-bound, phosphorylated state of the AT_1R recruits important regulatory proteins termed arrestins, which bind to, desensitize and cause internalization of the receptor (Thomas & Qian, 2003). Much remains to be determined in regard to the exact sites of phosphorylation, the hierarchy (or order) in which these residues are modified and what effect this has on selective coupling to specific signaling pathways.

3.1.1. G protein receptor kinases

The strength and duration of receptor signaling is crucially dependent on phosphorylation by members of the GRK family (Metaye et al., 2005). Seven mammalian genes, encoding GRK1-7, are subdivided into 3 groups: the rhodopsin family (GRK1/7), the Gβγ-binding, pleckstrin homology-containing GRK2/3, and the membrane-associated GRK4/5/6 (Kim et al., 2005). The expression of GRK1/7 (retina) and GRK4 (testes, brain, myometrium and kidney) are restricted, whereas the other GRK (GRK2/3/5/6) are widely distributed and regulate the hundreds of GPCR encoded in our genome (Metaye et al., 2005).

AT_1R can be phosphorylated by over-expressed GRK2/3/5 in transfected cell culture systems (Oppermann et al., 1996a,b), but the kinase(s) involved in vivo have not been determined. The relative contribution of the common GRK (GRK2/3/5/6) to AT_1R phosphorylation has also been examined by RNA interference (RNAi), using siRNA to selectively and individually knockdown the expression of each of these isoforms (Kim et al., 2005). In HEK293 cells, GRK2 appears to be the primary kinase mediating AngII-stimulated AT_1R phosphorylation, accounting for about 80% of the total GRK-mediated phosphorylation. Interestingly, in co-immunoprecipitation experiments, all the kinases (GRK2/3/5/6) contributed to the direct association between the AT_1R and β-arrestin 2, suggesting that arrestin recruitment does not necessarily correlate with the amount of phosphorylation. Such an idea was supported by the reciprocal involvement of GRK2/3 versus GRK5/6 in other arrestin-dependent processes. Thus, knockdown of GRK2/3, but not GRK5/6, significantly inhibited AT_1R internalization, whereas reduction GRK5/6, but not GRK2/3, impaired the arrestin-dependent, prolonged phase of AT_1R-mediated ERK activation (Kim et al., 2005). These data suggest that different patterns of receptor phosphorylation can be mediated by the different GRK and subserve distinct functions. Of course, such an interpretation is based on the assumption (that remains unfounded) that GRK exclusively phosphorylate activated forms of GPCR and no other substrate that might impinge on the processes examined.

3.1.2. Protein kinase C

PKC belongs to a family of phospholipid-dependent serine/threonine kinases (Nishizuka, 1986; Parker & Murray-Rust, 2004) which are involved in a variety of biological functions such as cellular signal transduction and its regulation through

[2] Human genome sequence: www.ncbi.nlm.nih.gov/genome/guide/human/.

phosphorylation of specific substrates. Various stimuli, including $G_{\alpha q/11}$-phospholipase Cβ signaling via GPCR, can activate PKC isoforms, and green fluorescent protein (GFP)-tagged versions of PKC can be used to visualize activation and translocation to and from the plasma membrane (Feng et al., 1998; Violin & Newton, 2003). In contrast to GRK, PKC phosphorylates GPCR in the absence of agonist stimulation; so PKC translocated to the membrane in response to activation by one particular receptor has the capacity to phosphorylate and regulate a spectrum of other cell surface proteins and receptors, including the signaling receptor itself. In the case of the AT_1R, 3 serine residues within its carboxyl-terminus (Ser^{331}, Ser^{338} and Ser^{348}) are in the consensus motif for PKC phosphorylation and all 3 are modified in response to PKC activation (Qian et al., 1999). PKC phosphorylation occurred mainly in response to low levels of AngII stimulation, whereas GRK phosphorylation of the AT_1R was more predominant at higher concentrations.

3.2. Arrestins

3.2.1. Structure–function of arrestin

Arrestins are cytoplasmic proteins that recognize and bind phosphorylated GPCR and regulate their function (for recent reviews, see Gurevich & Gurevich, 2004; Lefkowitz & Shenoy, 2005; Shenoy & Lefkowitz, 2005b; Gurevich & Gurevich, 2006). They terminate GPCR signaling by preventing further productive interaction with G proteins and promote internalization via interactions with components of the endocytic machinery (e.g., clathrin, AP-2, NSF, ARNO, ARF, microtubules). There are 4 main members of the arrestin family—2 are expressed exclusively in the retina (visual arrestin and cone arrestin) and act on photoreceptors; the other 2 non-visual arrestins, termed β-arrestin 1 and β-arrestin 2, share 78% homology, are ubiquitously expressed and can interact with hundreds of different GPCR. High-resolution crystal structures have been obtained for the basal "inactive" state of arrestin, which undergoes significant conformational rearrangement upon activation and interaction with phosphorylated receptors (Xiao et al., 2004; Charest et al., 2005; Gurevich & Gurevich, 2006; Hanson et al., 2006). The carboxyl-terminus of arrestin undergoes major displacement revealing several important binding sites, which presumably mediate endocytosis and other scaffolding functions.

GPCR are classified as Class A or Class B based on their proclivity to traffic β-arrestin 1 and β-arrestin 2 (Oakley et al., 2000). Class A receptors preferentially recruit β-arrestin 2 and are typified by the β_2-adrenergic receptor, whereas class B receptors, such as the AT_1R, robustly recruit both β-arrestins. In addition, class B receptors internalize as stable complexes with β-arrestins into endosomes, but the interaction of β-arrestins with class A receptors is transient, with the receptors translocating to endosomes and the β-arrestins partitioning back to the cytosol. Why GPCR traffic with or without β-arrestin, or have preference for one β-arrestin over another, remains to be clarified, although this seems to be related to the more recently described functions of arrestins (reviewed below). A molecular dissection of these issues is paramount—as briefly described below and reviewed comprehensively elsewhere (Lefkowitz & Shenoy, 2005; Shenoy & Lefkowitz, 2005b), arrestin-specific signals continue to emerge; their contribution to the biology of GPCR and other receptor systems is undeniable, while their broader actions (tolerance and addiction, chemotaxis, metastasis, retinal degeneration, cardiovascular dysfunction) are ultimately linked to the subtle differences that underlie the strength and "flavour" of β-arrestin recruitment, scaffolding and regulation.

3.2.2. Alternative functions of β-arrestins

The role of β-arrestins in signal termination is complemented by their capacity to act as scaffolds to recruit signaling/regulatory molecules to the receptor (Lefkowitz & Shenoy, 2005; Shenoy & Lefkowitz, 2005b). An impressive array of molecules has been reported to interact with arrestins, including kinases (c-Src, ERK, JNK, Akt), phosphatases (PP2A), cytoskeletal proteins (Ral-GDS, filamin), ubiquitin-ligases (Mdm2), lipids (inositol hexakisphosphate; Milano et al., 2006), sodium hydrogen exchangers (NHE5), transcriptional regulators (NF-kappaB inhibitor) and phosphodiesterases (PDE4D5). In this regard, arrestins have become almost as prevalent as the heterotrimeric G proteins in explaining the complexity of the GPCR function: they modulate olfaction, chemotaxis, apoptosis and survival (Merrill et al., 2002; Hunton et al., 2005; Merrill et al., 2005; Satoh & Ready, 2005); they can shuttle in and out of the nucleus and affect transcription and chromatin remodeling (Kang et al., 2005). Moreover, the actions of arrestins go beyond classical GPCR; they can bind and affect a variety of non-GPCR (low density lipoprotein receptor, transforming growth factor βII receptor, tumor necrosis factor receptor-associated factor 6, insulin-like growth factor-1 receptor) (Chen et al., 2003a; Wu et al., 2003; Girnita et al., 2005; Wang et al., 2006) and impinge on development through effects on *Frizzled* (Chen et al., 2003b), *Smoothened* (Chen et al., 2004; Wilbanks et al., 2004) and *Notch* (Mukherjee et al., 2005) signaling pathways.

4. G protein-independent signaling for type 1 angiotensin receptor

Conventional paradigms of GPCR activation and regulation evoke a single, monomeric receptor species that binds ligand and switches to an active state, which couples to G protein and generates intracellular signals. Receptor phosphorylation, arrestin binding and internalization then terminate these signals. While providing a useful framework for conceptualizing and investigating receptor biology, such models are now recognized as too simplistic. For example, an accumulating body of literature indicates that GPCR may actually exist and function as dimers or oligomeric complexes; GPCR can be activated even in the absence of ligand binding (e.g., by mutation or by physical perturbation of the cell); they can attain multiple functional states; they can exist as separate isoforms (via polymorphism or alternative splicing); and they can continue to signal after internalization by virtue of the scaffolding and localization properties afforded by the arrestins. It is fascinating that a significant proportion of these GPCR activities and signals may occur

independently of the traditional coupling to G protein and that these actions have important biological consequences.

Significant insights into such atypical receptor behaviour have stemmed directly from research into the AT_1R. This reflects the development and availability of some unique experimental tools with which to probe receptor function. These include (1) a panel of mutant AT_1R with distinctive features, including selective uncoupling from G proteins and a capacity to sustain other receptor functions, such as receptor phosphorylation, arrestin recruitment and internalization; (2) a substituted version of AngII, $Sar^1Ile^4Ile^8$-AngII, which binds the receptor but fails to activate G protein (specifically $G_{q/11}$) (Noda et al., 1996)—this substituted ligand does, however, promote receptor phosphorylation and arrestin recruitment and has been instrumental in elucidating G protein-independent events; (3) the application of RNAi approaches to selectively knockdown the expression of GRK, arrestins and G proteins as a means of unambiguously ascribing functional input has been invaluable. In a previous review (Thomas et al., 2004), we examined the evidence for G protein-independent actions for the AT_1R receptor and the concept of multiple receptor states that allow receptor signals and activities to be partitioned both temporally and spatially. In the following sections, we will provide some of this background information, but mostly focus on new information that has appeared in the last few years and its potential therapeutic relevance.

4.1. G protein-uncoupled type 1 angiotensin receptors

4.1.1. Receptor internalization

Some of the earliest evidence refuting the idea that AT_1R activation transits along a linear path – from ligand binding, through G protein activation, phosphorylation, arrestin binding and internalization – came from studies investigating the process of AT_1R-mediated endocytosis. Hunyady et al. (1994a) were the first to report that AT_1R mutants with severely inhizbited $G_{q/11}$ coupling retained robust AngII-induced receptor internalization, highlighting the possibility that different receptor states separately subserve receptor signaling and endocytosis. This observation has been confirmed by independent laboratories (Thomas et al., 2000; Miserey-Lenkei et al., 2001; Holloway et al., 2002; Gaborik et al., 2003) and extended to include evidence that mutant AngII ligands (like $Sar^1Ile^4Ile^8$-AngII) can promote receptor phosphorylation and internalization in the absence of $G_{q/11}$ signaling. Conversely, constitutively active AT_1R (receptor mutants that couple to $G_{q/11}$ in the absence of ligand binding) show very poor AngII-mediated phosphorylation, but display robust internalization (Thomas et al., 2000). Based on a number of such studies, it is now generally accepted that AT_1R activation is not necessarily a linear concatenation of mutually dependent events, but rather a series of interrelated, yet definably separate, receptor conformations, which underlie the various aspects of receptor function.

4.1.2. Signal transduction

Such $G_{q/11}$-uncoupled AT_1R mutants have also shed light on the subtle differences that discriminate the various signals emanating from the AT_1R. Thus, a series of receptor variants have been described (M5, $D^{74}E$, i2m, $D^{74}N$, $Y^{292}F$, $N^{295}S$; (Doan et al., 2001; Seta et al., 2002; Hines et al., 2003)) in which $G_{q/11}$ signaling is abrogated, yet coupling to other signals such as the JAK-STAT pathway, Src tyrosine kinase, and ERK activation are retained. The mechanism of G protein-independent coupling remains uncertain, but may involve distinct conformational states of the AT_1R, dictated by key residues within the 7th transmembrane helix (TM) (Yee et al., 2006), and/or direct interaction of these signaling components with the carboxyl-terminus of the AT_1R. For example, convincing evidence exists (outlined in Section 4.1.3) to support a role for arrestin binding to the AT_1R carboxyl-terminus as the mediator of ERK signaling by $G_{q/11}$-uncoupled AT_1R.

Physical association between the AT_1R and EGFR has also been reported (Seta & Sadoshima, 2003; Olivares-Reyes et al., 2005) and suggested as the mechanism of AngII-mediated EGFR transactivation (Seta & Sadoshima, 2003), a process whereby stimulation of GPCR leads to tyrosine phosphorylation of the EGFR and the subsequent activation of downstream growth signaling pathways, such as ERK and phosphoinositide 3-kinase (Fischer et al., 2003). EGFR transactivation contributes significantly to AngII growth effects in tumour cells (Schafer et al., 2004), the heart (Shah & Catt, 2004; Smith et al., 2004), kidney (Lautrette et al., 2005), vasculature (Suzuki et al., 2005; Nakashima et al., 2006) and skin (Yahata et al., 2006). In their paper, Seta and Sadoshima (2003) proposed that tyrosine^{319} in the AT_1R carboxyl-terminus is phosphorylated during receptor activation and this mediates an interaction with EGFR, which drives the ensuing transactivation. They also reported that when the interaction between AT_1R–EGFR was prevented (by mutation of tyrosine^{319} to phenylalanine, $Y^{319}F$), EGFR transactivation-dependent growth responses (ERK signaling and proliferation) were lost. It should be noted that, in direct contrast, Mifune et al. (2005) and Shah et al. (2004) recently reported that the $Y^{319}F$ AT_1R mutant was not defective and retained the capacity to transactivate the EGFR. Instead, the data point to a mechanism more likely to involve a $G_{q/11}$-mediated activation of the metalloproteinase, ADAM17, and the cell surface shedding of the EGF ligand (heparin-binding EGF-like ligand) and the resulting binding and activation of EGFR. This latter mechanism is more in keeping with prevailing theories on GPCR–EGFR transactivation (Fischer et al., 2003).

4.1.3. Arrestin-based actions

Without a doubt, a series of recent studies, primarily from Lefkowitz's group at Duke University (Lefkowitz & Shenoy, 2005; Shenoy & Lefkowitz, 2005b), have provided the most convincing data yet for G protein-independent signaling from the AT_1R. The arrestin proteins, most typically associated with signal termination and receptor desensitization and internalization, appear to concurrently act as major scaffolding molecules, particularly for ERK MAPK. This arrestin-mediated ERK activation is retained under conditions where G protein coupling from the AT_1R is inhibited and provides an intracellular pool of activated ERK that may have important biological consequences.

4.1.3.1. Recruitment and trafficking to activated receptors

The activated AT_1R is a class B GPCR (Oakley et al., 2000) that

recruits both β-arrestin 1 and β-arrestin 2, which form stable complexes that internalize and co-localize on endocytic vesicles. The internalization of AT_1R is strongly inhibited in cells lacking β-arrestin 1 and β-arrestin 2 (Kohout et al., 2001) or where arrestin expression has been depleted using RNAi (Ahn et al., 2003). The stability of AT_1R–arrestin interactions and the class B nature of their trafficking is dependent upon both the phosphorylation of the receptor within its carboxyl-terminus as well as the pattern of arrestin modification by ubiquitination—the post-translational addition of ubiquitin molecules onto lysine residues in proteins (Shenoy & Lefkowitz, 2003, 2005a).

Expanding on the idea that activated AT_1R still internalize in the absence of $G_{q/11}$ coupling (Thomas et al., 2000; Miserey-Lenkei et al., 2001; Holloway et al., 2002; Gaborik et al., 2003), Wei et al. (2003) reported that $G_{q/11}$ activation is also not required for β-arrestin 2 recruitment to the AT_1R. Using confocal microscopy to follow the trafficking of GFP-labeled β-arrestin 2, they observed arrestin translocation to endocytic vesicles following AngII activation of a $G_{q/11}$-uncoupled AT_1R mutant (DRY/AAY), a mutant that fails to generate inositol phosphates in response to AngII. β-Arrestin 2 trafficking was also achieved by stimulating the wild type AT_1R with the substituted AngII ligand, $Sar^1Ile^4Ile^8$-AngII, which shows no detectable inositol phosphate generation through the AT_1R. Thus, receptor phosphorylation (Holloway et al., 2002) and arrestin recruitment (Wei et al., 2003) appear to be independent of the AT_1R conformation necessary to activate its cognate G protein.

4.1.3.2. Arrestin-scaffolded signaling. During the process of AT_1R receptor activation and internalization, β-arrestin 2 acts to coordinate the formation of a multi-protein complex that includes ERK MAPK and its upstream activators (Luttrell et al., 2001). Overexpression of arrestin leads to reduced inositol phosphate generation, consistent with its role in signal desensitization, but conversely promotes an enhanced ERK activation; this ERK is sequestered with the AT_1R and arrestin within the cytoplasm on endosomes, which coincides with a decreased nuclear pool of activated ERK and diminished transcriptional responses (Tohgo et al., 2002). Depletion of β-arrestin 2 levels using RNAi significantly shifted the dose–response curve for AngII-mediated ERK activation to the right, indicating an important contribution of arrestin to the sensitivity of AT_1R-induced ERK signaling. In another study (Ahn et al., 2004b), this inhibitory effect was even more profound. As was the case for arrestin trafficking with internalized receptors (as mentioned above), the amount of activated ERK bound to arrestin correlates well with the stability of the AT_1R–arrestin interaction (Tohgo et al., 2003), which depends on the carboxyl-terminus of the receptor and its phosphorylation. Indeed, the inhibitory actions of β-arrestin 2 RNAi on AngII-induced ERK activation are nullified when AT_1R are mutated or truncated at the carboxyl-terminus to prevent arrestin interaction (Wei et al., 2004).

In their study detailing β-arrestin 2 trafficking with the AT_1R in a $G_{q/11}$-independent fashion, Wei et al. (2003) also reported that AngII stimulation of the uncoupled receptor (DRY/AAY) and treatment of the wild type AT_1R with $Sar^1Ile^4Ile^8$-AngII could yield significant ERK activation—about half the amount activated by AngII though the wild type receptor. Based on RNAi approaches, this "uncoupled" ERK stimulation was completely dependent upon β-arrestin 2, whereas in the absence of β-arrestin 2, AngII-induced ERK activation via the wild type receptor was predominantly through mobilization of PKC activity. Temporally, the G protein-dependent portion of the AT_1R-mediated ERK was rapid (maximum at 1–2 min) and transient and resulted in nuclear translocation of the activated ERK, whereas the G protein-independent ERK activation developed more slowly, was β-arrestin 2—bound and sequestered in endosomes and sustained for up to 90 min (Ahn et al., 2004a). Finally, there appears to be a clear difference between β-arrestin 1 and β-arrestin 2 with regard to AT_1R-activated ERK signaling (Ahn et al., 2004b): in contrast to β-arrestin 2, depletion of β-arrestin 1 using RNAi effectively enhances AngII-induced, as well as $Sar^1Ile^4Ile^8$-AngII driven, ERK signaling through the wild type receptor. One interpretation of this data is that β-arrestin 1 may be acting in a dominant-negative fashion to oppose β-arrestin 2-sequestered activated ERK.

The principal discriminator between G protein-dependent and β-arrestin 2-dependent (i.e., $G_{q/11}$ independent) ERK activation is that the former generates a nuclear transcriptional response, typically associated with ERK activation and growth programs, whereas the latter sequesters activated ERK in the cytoplasmic vesicles and prevents subsequent nuclear events. Although the in vivo consequences for cellular growth of separate, spatially and temporally distinct pools of ERK are not yet evident, it would not be too surprising if somatic ablation of β-arrestin 2 in animals might yield pro-growth outcomes. In Drosophila, one form of arrestin is considered apoptotic, whereas the other is pro-survival (Satoh & Ready, 2005). Whether a similar scenario exist for β-arrestin 2, with antagonism provided by β-arrestin 1 as indicated by the in vitro studies (Ahn et al., 2004b), awaits experimental verification. One functional outcome that has been directly related to an AT_1R–β-arrestin 2/$G_{q/11}$-independent signal is that of chemotaxis—the migration of cells in the direction of a chemical signal (Hunton et al., 2005). AngII mediated chemotaxis in HEK293 cells was mimicked by the uncoupled ligand, $Sar^1Ile^4Ile^8$-AngII, and chemotaxis to both AngII and $Sar^1Ile^4Ile^8$-AngII was abrogated by depletion of β-arrestin 2 by RNAi. Whether β-arrestin 1 can antagonize this chemotaxis is not clear, but β-arrestin 1 (and not β-arrestin 2) has been reported to act in co-ordination with $G_{q/11}$ to activate RhoA and cytoskeletal re-organization (Barnes et al., 2005).

4.1.4. In vivo and therapeutic implications

Few studies have yet to extrapolate the in vitro observation of G protein-independent AT_1R activation to the in vivo situation. Two very recent papers on brain (Daniels et al., 2005) and heart (Zhai et al., 2005) studies have provided early indications that distinct signaling pathways activated by AT_1R may have profound physiological and behavioral outcomes.

In the first study (Daniels et al., 2005), Fluharty et al. examined the well-established role of AngII in eliciting thirst and salt appetite when injected centrally in the rat. Based on its capacity to activate ERK in the absence of $G_{q/11}$-mediated

signaling, they tested the effect of the AngII analog, $Sar^1Ile^4Ile^8$-AngII, on drinking and salt intake. Their major findings were that $Sar^1Ile^4Ile^8$-AngII acted as an antagonist of AngII-mediated inositol phosphate signaling in vitro and AngII-induced drinking in vivo. On its own, $Sar^1Ile^4Ile^8$-AngII did not elicit a drinking response, but it did promote increased salt appetite. These data suggest that the immediate drinking response to AngII is a $G_{q/11}$-dependent mechanism, but that the more slowly developing increase in salt intake occurs via $G_{q/11}$-independent signals, presumably through arrestin-based ERK signaling.

In the other study, Zhai et al. (2005) generated transgenic mice with a cardiomyocyte-specific overexpression of either the wild type AT_1R or a mutated, G protein-uncoupled version of the receptor (termed i2m), which carries mutations in the conserved DRY sequence at the junction of the third TM and intracellular loop 2. This mutation is analogous to the DRY mutants used by other investigators in the various in vitro experiments detailed above. Given the established role of $G_{q/11}$ signaling in cardiac hypertrophy, it might have been reasonable to hypothesize that the i2m transgenic mice would show diminished or ablated AngII-mediated hypertrophy. Instead, the mice displayed a profound cardiac hypertrophy to an extent that was even greater than that observed in the wild type AT_1R transgenic mice, which were matched for equivalent levels of receptor expression. The i2m mice also displayed electrophysiological disturbances and developed more severe bradycardia with reduced cardiac function. Interestingly, compared to the wild type transgenics, the i2m mice showed less fibrosis and cell death, indicating that these actions of AT_1R are via $G_{q/11}$ pathways. Overall, their study highlights the possible contribution of G protein-independent activities of AT_1R in pathology. In their commentary that accompanied this paper upon its publication, Lefkowitz et al. (Rajagopal et al., 2005) provide compelling arguments and comparisons that suggest that the mechanistic basis for the G protein-independent events reported by Zhai et al. are closely aligned with the arrestin-scaffolding phenomenon.

4.2. Ligand-independent type 1 angiotensin receptor signaling

Evidence exists to indicate that GPCR can attain multiple, functionally active conformations, including those that allow constitutive signaling in the absence of ligand stimulation (Costa & Cotecchia, 2005). This can occur merely by overexpressing the receptor, but usually arises from an innate (or mutation-induced) proclivity to attain an active state. For the AT_1R, no naturally occurring mutations have been described that overtly produce constitutive activation of $G_{q/11}$-dependent signaling, although enhanced basal signaling has been reported in cells for AT_1R overexpression and engineered mutations (Noda et al., 1996). Instead, as outlined below, some evidence exists that physical distortion of a cell can activate the AT_1R in the absence of AngII binding (Zou et al., 2004) and this might make an important contribution to diseases like cardiac hypertrophy.

4.2.1. Stretch and type 1 angiotensin receptor activation

Increased hemodynamic load (pressure overload) on the heart causes robust hypertrophy (Frey & Olson, 2003). The mechanical stress associated with stretching of cardiomyocytes is thought to initiate these growth responses either directly or via release of trophic factors, such as AngII, endothelin, cytokines and transforming growth factor-β. Mechano-sensors, such as integrins, LIM proteins, the cytoskeleton and ion channels/exchangers, transmit the physical act of cellular stretching into typical growth-promoting signals (Sadoshima & Izumo, 1997; Ruwhof & van der Laarse, 2000), including $G_{q/11}$ activation, changes in calcium handling, MAPK, JAK/STAT (Pan et al., 1999), EGFR transactivation (Kippenberger et al., 2005) and Rho kinases (Aikawa et al., 1999). Under normal situations, this appears to require the AT_1R, because non-peptide receptor antagonists block pressure overload hypertrophy, while the components of the RAS are upregulated by stretch in vivo and in isolated cells (Malhotra et al., 1999) as a way of resetting the gain of the system. However, mechanical stretch-induced hypertrophy has been shown to persist in vivo and in isolated cardiomyocytes from $AT_{1A}R$ knockout mice, prompting the suggestion that the AT_1R is not absolutely essential (Harada et al., 1998; Kudoh et al., 1998). Moreover, various studies have reported that load-induced hypertrophy is mostly, partially, or not dependent on the release of AngII from cardiomyocytes in response to stretch.

In probably the most compelling study to date, Zou et al. (2004) reported that mechanical stress can indeed activate the AT_1R, but it does so through an AngII-independent mechanism. In their study, Zou et al. first confirmed that mechanical stretching of cardiomyocytes in culture could produce robust ERK activation (a hypertrophic signal), which was equivalent in strength to that observed when cells were stimulated with maximal concentrations of AngII. However, the level of AngII measured in the myocyte-conditioned media was very low and not significantly increased by stretch, indicating that AngII is unlikely to be the instigating factor. The stretch-conditioned media also failed to robustly activate a reporter cell line consisting of HEK293 cells stably transfected with the AT_1R. Interestingly, stretching these HEK293-AT_1R cells leads to readily demonstrable ERK activation, whereas it was not observed in naive HEK293 cells that do not express the AT_1R. A neutralizing antibody to AngII could not negate stretch-induced ERK in the HEK293-AT_1R cells, but it was completely blocked by the AT_1R antagonist, candesartan. Together, these data indicate that mechanical stretch can activate the AT_1R through a mechanism distinct from active secretion of its ligand and receptor engagement.

The authors went on to demonstrate that AT_1R bearing mutations that prevent AngII binding ($K^{199}Q$) lost the capacity to respond to exogenous AngII, but retained stretch-mediated ERK activation, whereas mutations in the second intracellular loop that allow ligand binding, but uncouple the receptor from G protein activation, displayed diminished activation of ERK following stretch. Finally, in angiotensinogen knockout mice (which have no possibility of generating angiotensin peptides), cardiomyocyte ERK activation in response to stretch and in vivo pressure overload hypertrophy was retained, unambiguously discounting a role for the AngII ligand in this process. While no molecular explanation for the mechanism of stretch-

induced AngII-independent AT_1R was provided, the possibility that the AT_1R receptor is physically complexed with integrins and other mechanosensors that are able to modify its active state is intriguing and worth further pursuit.

4.2.2. Receptor dimerization

An accumulating body of evidence from biochemical, fluorescent-based proximity assays and functional approaches now supports the idea that GPCR exist not strictly as monomers as originally conceptualized, but as dimers and oligomers (Rios et al., 2001; Terrillon & Bouvier, 2004; Milligan, 2004, 2006). Using atomic force microscopy – a physical technique that can reveal cell surface topography at the level of individual proteins – rhodopsin receptors in the retina were resolved as rows of paired receptor proteins indicative of dimerization (Fotiadis et al., 2003). Indeed, it has been estimated that the proportion of a receptor population that is dimerized at any given point might be as high as 80% (Mercier et al., 2002). Many GPCR have now been reported to form dimers with themselves (homodimerization) or with other GPCR (heterodimerization). However, the functional significance of receptor dimerization remains controversial primarily because receptor dimers commonly form independently of ligand binding, with agonist and/or antagonist occupancy doing little to modulate the physical association between 2 receptors. How such a circumstance might be reconciled with dynamic agonist-driven regulation of receptor function has been a major hurdle in the acceptance of this paradigm, but may point instead to subtle conformational changes.

Not surprisingly, the concept of AT_1R homo- and heterodimerization has attracted considerable attention and controversy, not just because of the evidence that AT_1R dimers might possess altered pharmacological properties, but more so because recent studies have reported direct links between dimerized AT_1R pairs and pathology.

4.2.2.1. Biochemical and biophysical evidence. Over 20 years ago, Rogers (1984) first reported a possible homodimeric state of the AT_1R. Using high affinity radioligand labeling on myocardial sarcolemmal membranes, agonist (but not antagonist) ligand was cross-linked to an angiotensin receptor-binding site (note, cloning of the AT_1R was still a number of years away). SDS/PAGE analysis indicated that the receptor from the membrane existed as a noncovalent dimer (~116 kDa) equivalent to approximately 2 subunits, each with molecular weight of 60 kDa.

In 1996, following the cloning of the AT_1R and their firm establishment as the major protagonists of AngII actions, Eric Clauser's group was generating various AT_1R mutants and using these in structure-function studies to probe the binding site for AngII (Monnot et al., 1996). Two AT_1R mutants ($K^{102}A$ within TM3 and $K^{199}A$ within TM5) were identified that expressed at the cell surface but failed to bind AngII. Remarkably, when these binding-deficient receptors were co-expressed in cells, high affinity binding of AngII was restored. This functional trans-complementation provided evidence for a potential intermolecular interaction between AT_1R pairs. Interestingly, AngII stimulation of cells co-expressing $K^{102}A$ and $K^{199}A$ failed to produce a G_q-mediated signal, an observation that would be consistent with future studies on other GPCR, where dimerized receptors are not necessarily ligand-responsive.

Most recently, Hansen et al. (2004b) demonstrated the existence of homodimerized AT_1R in living cells by using the approach of bioluminescence resonance energy transfer (BRET). In this technique (for review, see Pfleger et al., 2006; Pfleger & Eidne, 2006), close proximity between protein partners (in this case putative receptor dimers) can be examined by fusing the receptors separately to either a luciferase enzyme or a modified form of GFP. Cells co-expressing the receptor fusions are treated with a substrate for the luciferase enzyme, which emits at a wavelength that excites GFP; if the GFP is in close enough proximity to absorb the energy, as would the case if the receptor pairs are dimerized, then it would emit at its characteristic wavelength and a BRET readout would be obtained. Using this approach, Hansen et al. (2004b) noted the constitutive formation of AT_1R homodimers, which were not formed at the cell surface but rather during biosynthesis as the receptors trafficked through the endoplasmic reticulum; the constitutive nature of receptor dimerization was not affected by treatment with agonists or antagonists. Nevertheless, as discussed in Section 4.2.2.3, this dimerization reportedly promoted distinct receptor conformations with specific signaling outcomes.

The first example of AT_1R heterodimerization was reported by AbdAlla et al. (2000) between the AT_1R and the bradykinin B2 receptor. The heterodimer was originally observed in A10 smooth muscle cells, which endogenously express both receptors, using a ligand affinity-labeling approach combined with immuno-affinity chromatography. Either receptor could be enriched with the other using their respective antibodies. Their physical association was confirmed by co-immunoprecipitation of the endogenous receptors from the smooth muscle cells or in HEK293 cells co-expressing differentially epitope-tagged versions of the receptors. Subsequently, using a variety of approaches, heterodimerization has been reported between AT_1R–AT_2R (AbdAlla et al., 2001a), AT_1R–β2-adrenegic receptor (Barki-Harrington et al., 2003), AT_1R–dopamine D5 receptor (Zeng et al., 2005) and AT_1R–Mas (Kostenis et al., 2005) (see Table 1).

4.2.2.2. Molecular mechanisms of dimerization. Although the formation of AT_1R homo- and hetero-dimers is constitutive and mostly ligand-independent, a level of specificity does seem to exist. For example, in BRET-based assays, the AT_1R homodimerizes, but does not heterodimerize with other GPCR, such as the endothelin ET_A or the calcium-sensing receptor (Hansen et al., 2004a), indicating the specificity of the interaction and confirming that over-expression of 2 proteins in plasma membrane is not sufficient to force a BRET signal. Similarly, the Mas receptor causes a constitutive BRET signal with the AT_1R, but not the thyrotropin-releasing hormone receptor (Kostenis et al., 2005). At least for AT_1R homodimers, the physical interaction between receptor pairs apparently happens during the process of biosynthesis and prior to export from the endoplasmic reticulum and subsequent trafficking to

Table 1
AT_1R receptor dimerization

Dimer pair	Techniques used	Predicted function	Reference
AT_1R–AT_1R	SW	↑ signaling, atherosclerosis	AbdAlla et al., 2004
AT_1R–AT_1R	B	Constitutive dimers; if one of the dimer pairs is defective in binding or activation, Gq signaling is inhibited but ERK is retained	Hansen et al., 2004b
AT_1R–B2 bradykinin	SW, C	↑ signaling, preeclampsia	AbdAlla et al., 2000, 2001b
AT_1R–β2 adrenergic	C	Constitutive dimers, cross-inhibition	Barki-Harrington et al., 2003
AT_1R–D5 dopamine	L	AT_1R down-regulates D5 and D5 expression down-regulates AT_1R	Zeng et al., 2005
AT_1R–Mas	C, B	Mas upregulates AT_1R expression, but decreases signaling	Kostenis et al., 2005; Canals et al., 2006
AT_1R–AT_2R	SW	AT_2R expression acts to antagonize AT_1R signaling	AbdAlla et al., 2001a

Techniques used: SW, SDS-PAGE/Western blotting; C, co-immunoprecipitaiton; B, BRET; L, colocalization.

the cell surface (Hansen et al., 2004b). Whether this holds for other AT_1R dimer partners remains to be determined, but the regulated secretion/aggregation technology employed by Hansen et al. (2004b) would be particularly useful in examining this possibility.

In what can only be described as a major paradigm shift, AbdAlla et al. (2004) proposed an entirely new mechanism for GPCR dimerization. In contrast to prevailing theories (Milligan, 2004), which evoked disulphide linkages between adjacent receptor pairs or direct interactions between their transmembrane helices, Ursula Quitterer et al. (AbdAlla et al., 2004) reported that, in activated monocytes, AT_1R dimerization is catalyzed by an intracellular enzyme, factor XIIIA transglutaminase, which can covalently crosslink proteins via glutamines on one protein and lysines on another. Mutations of glutamine^{315} in the cytoplasmic carboxyl-terminus of the AT_1R abolished dimer formation identifying it as the likely residue targeted by the transglutaminase. This study was remarkable for 2 reasons: (1) because dimerization was agonist-induced and (2) as detailed in Section 4.2.2.4, AT_1R dimers showed enhanced signaling and seemed to directly contribute to the development of atherosclerosis. Whether transglutaminase-mediated dimerization of AT_1R can be independently confirmed and if it represents a general mechanism for GPCR dimer formation awaits further investigation.

4.2.2.3. Effects on ligand affinity, signaling and receptor trafficking. The capacity of AT_1R dimerization to alter ligand binding and consequently affect efficacy and potency to either potentiate or attenuate relative G protein-coupled signaling has been reported. For example, AT_1R/B2 receptor heterodimerization induced a positive-cooperative effect on the AT_1R, promoting increased ligand affinity and resulting in enhanced AngII-induced G protein activation and inositol phosphate production. In a way, this mirrors the enhanced AngII-mediated signaling reported for the transglutaminase cross-linked AT_1R homodimers in monocytes (AbdAlla et al., 2004). In contrast, the B2 bradykinin receptor in the AT_1R–B2 dimer showed reduced ligand affinity and decreased signaling upon bradykinin stimulation. Interestingly, the dominant-positive effect on AT_1R signaling exerted by the AT_1R–B2 heterodimers did not require high affinity binding of the B2 receptor, but was dependent on the integrity of B2 receptor/G protein coupling. In addition, the formation of AT_1R–B2 receptor dimers appeared to alter the mechanism by which both receptors endocytose—when expressed alone, the internalization of both receptors was insensitive to a dominant-negative version of dynamin (a protein that pinches off coated vesicles to permit internalization), whereas co-expression of the AT_1R and B2 receptor led to dynamin-dependent endocytosis of both receptors.

In contrast, the heterodimers formed between AT_1R–AT_2R and AT_1R–Mas appear to antagonize the $G_{q/11}$-mediated signaling of the AT_1R (AbdAlla et al., 2001a; Kostenis et al., 2005). Thus, titration of the AT_2R into cells co-expressing the AT_1R led to a graded decrease in AngII-mediated inositol phosphate generation. Remarkably, this antagonism was not related to AngII binding to the AT_2R, because the AT_2R-selective non-peptide inhibitor, PD123319, did not prevent it nor was this antagonism lost for AT_2R mutants defective in AngII binding. Similarly, co-expression of Mas with AT_1R significantly reduced the potency and maximal efficacy of AngII to mobilize intracellular calcium through AT_1R–$G_{q/11}$ coupling (Kostenis et al., 2005), even though Mas expression caused a paradoxical increase in AT_1R expression. Again, the effects of Mas were not modulated by its putative ligand, the AngII fragment, Ang1-7 (Santos et al., 2003). Finally, in Mas knockout mice, AT_1R vasoconstriction was enhanced, consistent with an antagonistic role for Mas in a physiological setting. While these data strongly support the idea that direct AT_1R–Mas heterodmerization results in functional antagonism of the AT_1R, a recent follow-up study has suggested that Mas can constitutively activate $G_{q/11}$ signaling and thereby lead to PKC mediated phosphorylation of the AT_1R (Canals et al., 2006). So perhaps some of the inhibition of AngII signaling observed in the earlier study might relate to phosphorylation-mediated AT_1R desensitization.

A final example of receptor crosstalk that relates to AT_1R heterodimerization comes from the study of Barki-Harrington et al. (2003). In mouse cardiomyocytes, which co-express AT_1R and β-adrenergic receptors, pharmacological blockade of β-adrenergic receptors with propranolol abolished AngII stimulation of contractile responses. This antagonism did not result from interference with AngII binding to AT_1R, but rather through uncoupling of the AT_1R from $G_{q/11}$. Conversely, AT_1R antagonism with the specific, non-peptide antagonist, valsartan, prevented productive coupling of βadrenergic receptors to G_s in cardiac membranes and abrogated isoproterenol-stimulated cAMP generation in cells expressing both AT_1R and β-adrenergic receptors. AngII- and isoproterenol-activated ERK signaling and receptor-mediated internalization was also transinhibited by reciprocal antagonism. This phenomenon extended to the in vivo situation, where a single dose of

valsartan in mice markedly reduced the isopreterenol-induced increases in heart rate. The exact mechanism of this transinhibition remains unresolved but it may involve dimerization since the 2 receptors could be co-immunoprecipitated together from cells in a constitutive (ligand-independent) manner. Given that many of the hundreds of GPCR encoded by our genome are co-expressed in various cells, it will be of interest to determine how widespread this transinhibition phenomenon is and whether dimerization is the mechanism at play.

4.2.2.4. Receptor dimers and disease. Two seminal papers have linked AT_1R dimerization with cardiovascular-related disease, namely preeclampsia and atherosclerosis (AbdAlla et al., 2001b, 2004). Preeclampsia is a relatively common form of hypertension and proteinurea that occurs during pregnancy and is associated with increased sensitivity to AngII. Some have suggested that autoantibodies are generated against the extracellular regions of the AT_1R that act as agonists and increase signaling (Wallukat et al., 1999; Dechend et al., 2004; Shah, 2006). Quitterer et al., on the other hand, reported increased AT_1R–B2 bradykinin receptor heterodimers in platelets and isolated vessels from women with preeclampsia compared to normotensive pregnant patients. Dimerized receptors displayed increased responsiveness to AngII, which required the G protein activation domain of the B2 receptor, and the AT_1R in the dimer showed a decreased sensitivity to inactivation by oxidative stress. The implication from these results is that increased AT_1R–B2 receptor dimerization is a prelude to preeclampsia.

In the second study, homodimers of AT_1R with increased signaling capacity have been associated with the processes that initiate atherosclerosis (AbdAlla et al., 2004). Thus, in hypertensive patients and apolipoprotein E knockout mice (a model of hypercholesterolemic atherosclerosis), monocytes display an increased AngII-dependent adherence to the endothelium. These monocytes had increased ACE activity as well as increased transglutaminase activity (as mentioned earlier), which functions to cross-link monomeric AT_1R into dimers in hypertensive patients and dyslipidemic mice, but not in normotensive people or wild type mice. The dimerized receptors had increased $G_{q/11}$-mediated inositol phosphate signaling and prevention of this dimerization in patients and mice reversed the atherosclerotic phenotype.

4.2.2.5. Perspective. Despite all the research described above and the accumulating evidence supporting a role for dimerized AT_1R_s in altered receptor activity and signaling, much remains to be established with regard to the exact molecular steps involved and the rationale for G protein engagement and activation by the dimeric interface. AT_1R has been documented to be able to physically interact and functionally associate with a variety of accessory, scaffold, and signaling proteins to fulfil its various biological functions. However, molecular links between AT_1R dimerization and these proteins remain unproven. It has been suggested that β-arrestin could function as a bridge for GPCR dimerization (Milligan, 2004). While there is a well-demonstrated physical interaction of AT_1R with β-arrestin, little is known about the contribution of β-arrestin to the process of

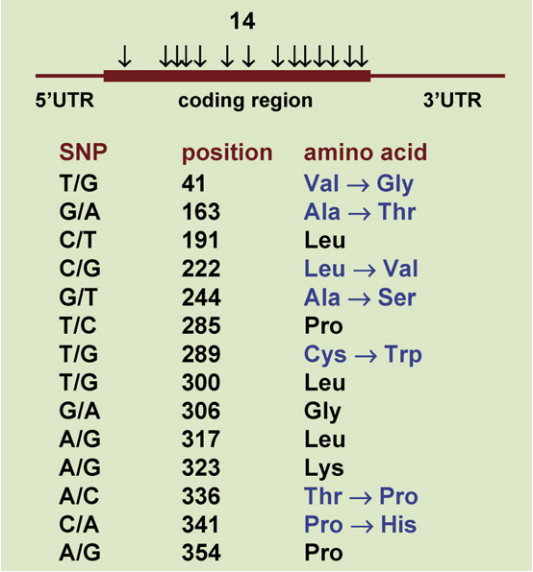

Fig. 2. Single nucleotide polymorphisms in the human AT_1R. The 1080 bp coding region of the human AT_1R has 14 putative SNP (see ncbi.nlm.nih.gov/SNP) indicated by arrows. Listed are the nucleotide variations (SNP), their corresponding amino acid position and whether the SNP is silent (synonymous) or results in an alteration in a particular amino acid in the receptor (nonsynonymous change, blue/gray).

AT_1R dimer formation, their regulation or conversely the effect of dimers on arrestin structure and function.

Clearly, the correlation between putative receptor dimers and diseases such as preeclampsia and atherosclerosis is provocative, but awaits independent verification. What is unarguable is that such observations do stimulate an interest in understanding new mechanisms for GPCR activation and regulation as well as the possibility of developing therapies that specifically target unique aspects of receptor biology.

4.3. Type 1 angiotensin receptor polymorphisms

The single-copy human *AGTR1* spans at least 45 kbp and contains 4 exons (the entire coding region is found in exon 4). Alternative splicing of exons 1, 2 and 3 onto exon 4 yields 4 main transcripts with markedly different rates of translation, indicating that mRNA processing may play an important role in determining the level of AT_1R expression. In addition, exon 3 encodes an in-frame 32 amino acid extension to the N-terminus producing a "long" isoform of the receptor with reduced affinity and signaling compared to the "short" form (Elton & Martin, 2003). The functional and clinical significance of long and short versions and other alternative splicing events of the AT_1R remains unclear, but it offers the cell choices with which to regulate AT_1R expression and function in a tissue-specific manner.

4.3.1. Evidence for variations in AGTR1

Single nucleotide polymorphisms (SNP) are individual nucleotide variations (e.g., C to T; G to A) within the genome. Current estimates predict that a SNP occurs every 100–300 base pairs along our 3 billion base pair genome; they occur within

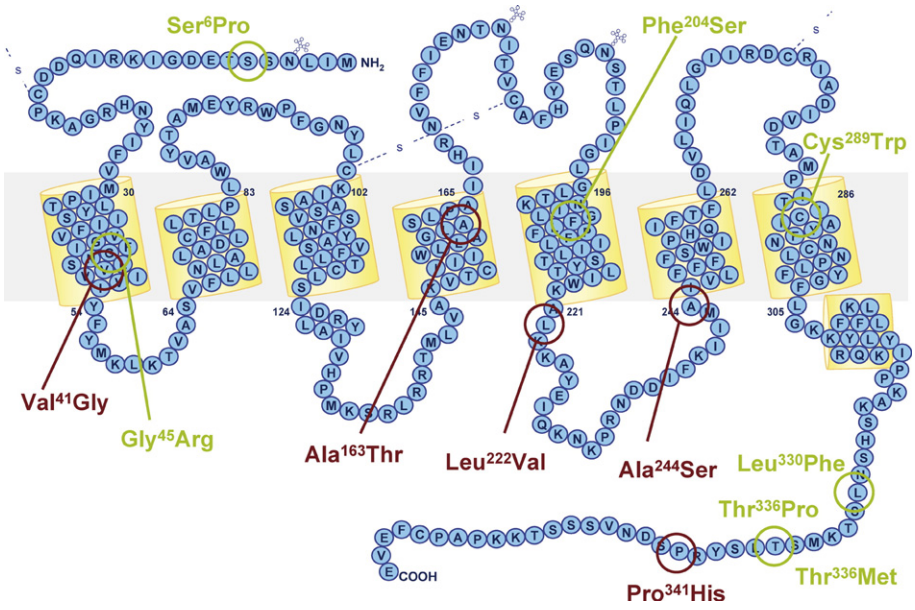

Fig. 3. Putative nonsynonymous polymorphisms in the human AT_1R. Shown is the 7 transmembrane-spanning topology of the 359 amino acid human AT_1R; standard single letter coding for the amino acids is used. The N-terminus is extracellular and the carboxyl-terminus is intracellular. Nonsynonymous SNP yielding amino acid changes in the human AT_1R are circled in purple or green, the latter indicating those tested pharmacologically by Hansen et al. (2004a).

exons (protein coding), introns (non-coding) and intergenic sequences. Presumably, the majority are functionally inert, although those in exons have the potential to change the encoded protein and thereby modify function; others may change gene transcription, RNA processing, splicing and/or stability, or protein translation. SNP potentially influence our susceptibility to disease and responsiveness to therapy and SNP maps are being developed to investigate the contribution of multiple genes to polygenic disorders, such as hypertension, cancer and diabetes.

The SNP database[3] lists over 600 putative SNP in the human *AGTR1*. Few of these have been validated nor their frequency of occurrence in populations established and even fewer have been functionally characterized or clearly linked to a physiological or pathological outcome. One of the most widely studied AT_1R SNP with regard to disease association is A1166C, which occurs in the 3′-untranslated region of the gene. It was first reported by Bonnardeaux et al. (1994) to associate with hypertension and to synergize with ACE polymorphisms for an increased risk of myocardial infarction (Tiret et al., 1994). At least 14 SNP are located in the coding region of the AT_1R and these are listed in Fig. 2. Seven of these SNP are nonsynonymous, in that an amino acid is changed, and these are depicted in Fig. 3. Also shown on Fig. 3 are additional polymorphisms examined by Hansen et al. (2004a), including $Ser^{6}Pro$ in the extracellular amino-terminus, $Gly^{45}Arg$ in TM1, $Phe^{204}Ser$ in TM5, and $Leu^{330}Phe$ in the cytoplasmic carboxyl-terminus. Whether these represent true polymorphisms or reflect changes induced by RNA editing or PCR-introduced alterations remains to be clarified as does their accurate frequency in the population. What is clear is that some of these polymorphisms have profound effects on receptor function.

[3] ncbi.nlm.nih.gov/SNP.

4.3.2. Functional implications

The nonsynonymous changes in the AT_1R (see Fig. 3) are located in the extracellular, transmembrane and intracellular regions, indicating that they may possibly perturb receptor binding, expression, activation and regulation. $Ser^{6}Pro$ might be expected to have some effect on the post-translational modification of the receptor by glycosylation because it changes a consensus motif (NXS/T) for N-linked glycosylation, which is known to modify the AT_1R and regulate cell surface expression (Deslauriers et al., 1999; Jayadev et al., 1999; Lanctot et al., 1999). Changes within the transmembrane domains ($Val^{41}Gly$, $Gly^{45}Arg$ in TM1; $Ala^{163}Thr$ in TM4; $Phe^{204}Ser$ in TM5; $Cys^{289}Trp$ in TM7) might be predicted to affect ligand binding or the conformational changes in the receptor that underlie activation and precede coupling to intracellular signaling molecules. The substitutions ($Leu^{222}Val$ and $Ala^{244}Ser$) in the N-terminal and C-terminal portions of the third intracellular loop could reasonably modulate coupling to G proteins, which are known to interact with the receptor at this site for many GPCR. Finally, changes within the carboxyl-terminal region ($Leu^{330}Phe$; $Thr^{336}Pro$; $Pro^{341}His$) might alter receptor desensitization, internalization or trafficking, specifically $Thr^{336}Pro$, which falls within the so-called "STL" motif—a site known to be phosphorylated, bound by arrestins and to contribute strongly to receptor internalization (Hunyady et al., 1994b; Thomas et al., 1998; Qian et al., 2001; Thomas & Qian, 2003).

Somewhat surprisingly, given the ascribed importance of the AT_1R to homeostasis and disease, few studies have directly studied the effect of polymorphisms in the *AGTR1* on AT_1R pharmacology and function. The study by Hansen et al. (2004a) is important because it compared the expression, binding and signaling of 7 AT_1R SNP when expressed ectopically in COS-7 monkey kidney cells and reported some

interesting findings. In a cell-based signaling readout, 3 of the variant receptors ($Gly^{45}Arg$; $Phe^{204}Ser$; $Cys^{289}Trp$) showed significantly decreased responses to agonists and antagonists, whereas the other 4 mutant receptors displayed wild type-like receptor activity. $Cys^{289}Trp$ displayed an 11-fold decrease in potency to AngII, but retained full maximal activity; $Phe^{204}Ser$ had a >50-fold reduction in potency with a 40% decrease in efficacy compared to wild type; whereas $Gly^{45}Arg$ shown a completely abrogated response to AngII. The inhibitory capacity of non-peptide AT_1R antagonists was also blunted for $Phe^{204}Ser$ (~20-fold) and $Cys^{289}Trp$ (~10-fold). Whole cell radioligand binding assays and fluorescent detection of receptors demonstrated that the $Gly^{45}Arg$ was expressed at the cell surface, but completely lacked the capacity to bind AngII, whereas $Phe^{204}Ser$ and $Cys^{289}Trp$ had lower expression and affinity for ligand. Not surprisingly, therefore, inositol phosphate accumulation and ERK1/2 activation in response to AngII stimulation of these receptors was either reduced ($Phe^{204}Ser$ and $Cys^{289}Trp$) or completely abolished ($Gly^{45}Arg$).

The validation of these functionally compromised AT_1R variants and their frequency in the general population requires larger studies and further analyses. Preliminary surveys suggest frequencies of up to 5% for $Gly^{45}Arg$ (Rolfs et al., 1994), but this needs verification. So, what would be the outcome for a person carrying this mutation? Homozygous penetrance would yield an effective AT_1R knockout individual, presumably with many of the renal abnormalities and blood pressure control issues observed in AT_1R knockout rodent models (Oliverio et al., 1998). Even carrying the variants, $Phe^{204}Ser$ and $Cys^{289}Trp$, would leave a person with a significantly reduced capacity to respond to AngII. Heterozygotes might expect normalcy if the wild type allele and the variant receptor proteins functioned independently, but what if these variants have the capacity to dimerize with the wild type and act as modifiers or dominant negative inhibitors? The effect of co-expressing these mutants with the wild type receptor was not attempted by Hansen et al. (2004a), but is worth considering given the accumulating evidence for functional receptor–receptor dimerization and interactions (Section 4.2.2.3).

4.3.3. Type 1 angiotensin receptor polymorphism and disease

The initial studies, reporting the association of the A1166C AT_1R polymorphism with high blood pressure (Bonnardeaux et al., 1994), heart attack (Tiret et al., 1994) and aortic stiffness (Benetos et al., 1996), generated a tremendous amount of interest in investigating possible links of this particular SNP with disease. Subsequent studies over the last decade have proven less convincing, with some studies reporting associations of A1166C with a range of cardiovascular disorders and others failing to do so (Miller & Scholey, 2004; Baudin, 2005). The link between the genotype and its phenotypic expression appears to be influenced by race, gender, underlying physiological perturbations (e.g., salt imbalance) and co-existing SNP in angiotensinogen and ACE. That A1166C resides in the 3′-untranslated region of the gene means it does not change the amino acid sequence of the receptor and so how it affects receptor function may involve changes in mRNA stability or processing. The association of the other AT_1R SNP with disease awaits further investigation, particularly the nonsynonymous ones that display altered expression and pharmacology.

5. Conclusions

The RAS continues to evolve in complexity. The net result of any stimulus necessarily involves the balance between the primary actions of AngII on AT_1R and the activities of AngII and other angiotensin fragments on additional receptor types. They are complicated by the dimerization of angiotensin receptors and cross-regulation of their signal transduction pathways as well as additional processing and signaling capabilities recently attributed to renin and ACE/ACE2. They may reflect actions of angiotensin through a "wild type" receptor or one of the many variants that exist. Even in the reductionist's view of AngII-stimulated activation of a single AT_1R species, one must consider actions mediated by the G protein as well as parallel functions driven by G protein-independent mechanisms. These may be synergistic or counteractive and might make important contributions to health and disease. A major challenge for researches in this area involves assimilating a vast array of literature and being able to integrate that information with their own data. The correct interpretation of experimental outcomes is acutely dependent upon a solid appreciation of the complex processes involved in angiotensin production, reception, signaling and regulation.

Some major issues remain: If the AT_1R receptor can be activated in the absence of ligand as reported for stretch-induced cardiac hypertrophy (Zou et al., 2004), what is the mechanism? Does it involve post-translational modification of the receptor (e.g., phosphorylation) or are AT_1R physically linked to the mechanosensors and, if so, how do they crosstalk? Real advancement will require careful dissection of the intricate receptor-networking complexes that obviously exist at the cell surface; better techniques are required for probing the active state(s) of GPCR and relating those to functional outcomes.

Receptor dimerization has emerged as a major focus for receptor biologists, but do homo- and hetero-dimers really reflect functional units with altered pharmacology? Some provocative data exist and these tend to favour such a hypothesis. What does this mean therapeutically? The cross-inhibition reported between the AT_1R and β-adrenergic receptors (Barki-Harrington et al., 2003) provides a note of caution when interpreting the outcomes of "selective" receptor antagonism in vivo and more examples of such a phenomenon are likely to arise. The reported dimerization and antagonism of AT_1R by AT_2R (AbdAlla et al., 2001a) is nicely aligned with the prevailing notion that these receptors counteract each other's activities, yet we did not observe any obvious antagonism in isolated cardiomyocytes infected with AT_1R and AT_2R adenoviruses (D'Amore et al., 2005). While many are willing to accept the physical reality of receptor dimers, much work is still required to determine their relevance to receptor biology and disease.

Finally, just how many AT_1R variants do we have? Alternative splicing and SNP variations exist, but their prevalence in the population, their affect on receptor pharmacology and relevance to RAS activity remain under-appreciated. The 3 interesting

variants reported by Hansen et al. (2004a) are all loss-of-function mutations and no gain-of-function variants, which might relate better to an overactive RAS, were reported. Further pharmacological characterization of human AT_1R polymorphisms is required. Specifically, variants in the carboxyl-terminus of the receptor at position threonine^{336}, which alter a well-established motif for receptor phosphorylation, arrestin binding and recruitment and internalization, might be expected to show enhanced receptor activation as a consequence of diminished receptor desensitization. Conversely, they might rationally be expected to have reduced arrestin-based (potentially $G_{q/11}$-independent) signaling. Whether these receptor variants have altered signaling and what effect, if any, this has on the modulation of long-term AngII activity needs testing experimentally.

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