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G Protein–Coupled Receptors as Disease Targets: Emerging Paradigms

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The G protein–coupled receptor (GPCR) superfamily represents the largest class of mammalian cell surface receptors; approximately 1,000 genes (or 1% of the genome) encode members of this family. These receptors are the targets of many clinically important drugs; in fact, approximately half of all prescription drugs are targeted to this group of plasma membrane receptors. That being said, drugs have been developed for few GPCRs, and this field has tremendous potential for drug discovery. Marketed drug designs include GPCR agonists (eg, sumatriptan succinate, targeted to the hydroxytryptamine or serotonin receptor) and antagonists (loratadine, targeted to the histamine receptors).

BIOLOGY OF GPCRs

By definition, GPCRs possess 7-transmembrane domain signatures (weaving back and forth across the plasma membrane 7 times) and interact with membrane-associated small G proteins to mediate downstream signal transduction [eg, angiotensin II (Ang II) subtype 1 receptor (AT_1R) (Figure 1)]. In the simplest terms, an extracellular ligand (like Ang II, a ''first'' messenger) can interact with the extracellular or transmembrane domain of the receptor. Changes in the receptor conformation trigger dissociation of the G protein complex, and the active subunits of the G protein can stimulate production of second messengers [eg, these include 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) for Ang II]. IP3 triggers release of intracellular calcium stores, which can activate a number of intracellular enzymes, including protein kinases and microtubule-associated proteins. DAG activates protein kinase C, which in turn phosphorylates serine and threonine residues on several protein substrates, thereby modifying their activities. The specific second messengers generated depend on the particular genus of G protein that interacts with the receptor and, therefore, are dependent on the receptor protein sequence.

The G protein cascade design is a marvelous engineering feat in view of the fact that it allows for tremendous signal amplification from the cell surface. A single receptor-ligand complex may activate 10 or more G protein molecules before dissociation or receptor internalization. Each of the G proteins may in turn activate several effectors (adenylate cyclase or phospholipase C). The effectors ultimately can produce many second messengers. As a consequence of this cascade, it is estimated that 1 bound formyl peptide receptor on a neutrophil may generate 100 to 1,000 molecules of IP3 and as many as 10,000 molecules of free calcium in less than 1 minute.[1] Clearly, blocking (or stimulating) cell surface GPCRs with pharmaceutical antagonists (or agonists) can have impressive downstream consequences.

For the GPCRs, cell surface signaling is terminated by a series of fairly well-defined events. Binding of agonist may sequentially activate multiple G proteins until the receptor desensitizes to agonist exposure. Desensitization generally involves receptor phosphorylation by GPCR kinases, leading to recruitment of β-arrestin and targeting the receptor for internalization. Early on, it was thought that signaling from a GPCR stopped following internalization. It is now believed that signaling may continue through an alternative β-arrestin–dependent pathway. The fate of internalized receptors follows at least 3 pathways. First, some of all internalized receptor species are targeted for degradation via the lysosome pathway. Of the remaining, most receptor species associate only transiently with β-arrestin. For this population, following endosome internalization, β-arrestin dissociates at or near the plasma membrane, and the

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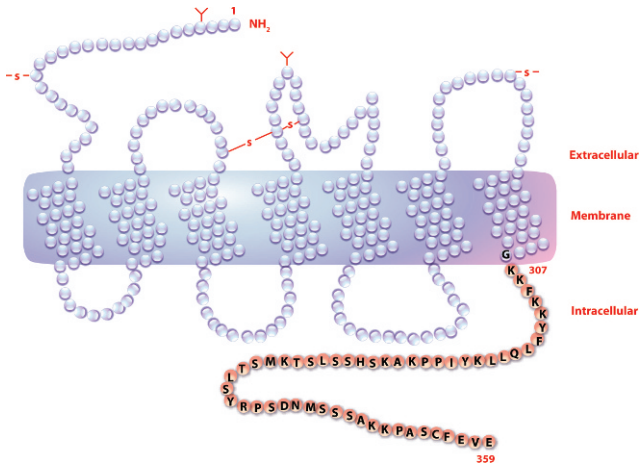

Figure 1. Rat AT_1 GPCR as it is oriented in the plasma membrane.

receptors return to the membrane, earning the designation of the rapid recycling pathway population. Other receptors such as the vasopressin 2 (V2) receptor and a subpopulation of the AT_1R internalize as a complex with β-arrestin and are recycled more slowly. It has become clear that β-arrestin, associated with these populations of receptors, can act as a scaffolding protein to link activated GPCRs to other signaling pathways, including c-Src, extracellular signal–regulated kinase 1 and 2 (ERK1/2), p38 mitogen-activated protein kinases (MAPKs), and c-Jun N-terminal kinase 3 (JNK3) in a G protein–independent manner (Figure 2). In other words, although β-arrestin proteins were discovered in the context of reducing receptor signals, it is now appreciated that they can initiate signals from the very receptors that they ''desensitize.''[2] This permits signaling to continue after receptor internalization into endosomes. Presumably, this occurs primarily in the endosomes of the slow recycling pathway. We believe that pathway coincides with the large endosomes described by Hunyady and colleagues[3] as the Rab-11 positive perinuclear recycling compartment. We also reported that this compartment shares membranes with the Golgi apparatus,[4] the compartment responsible for posttranslational modifications such as glycosylation. Because the Golgi is a processing site, it is tempting to speculate that a second reason for this recycling pathway may be to permit restoration of damaged receptors.

REGULATED INTRAMEMBRANE PROTEOLYSIS

Arguably, the most innovative and exciting work at the receptor forefront focuses on the idea that some conventional plasma membrane receptors also accumulate within cell nuclei (within the nuclear membrane or nucleosol) and that others undergo regulated intramembrane proteolysis (RIP) to produce receptor fragments that can continue to function within (or outside) cells to mediate biologically relevant events.[5–7] This principle has been demonstrated for many receptor tyrosine kinases and other single-pass (cross the membrane only once) membrane receptors. The epidermal growth factor receptor has been identified in the nucleus as an uncleaved ''holoprotein,'' while ErbB4, a member of the epidermal growth factor receptor family of tyrosine kinases, is perhaps the prototype receptor for RIP. When induced by activators such as tissue plasminogen activator, the tumor necrosis factor converting enzyme metalloprotease cleaves the extracellular domain of ErbB4, after which the enzyme complex, γ-secretase, cleaves within the transmembrane domain to generate an intracellular cleavage fragment that accumulates in the nucleus and presumably has nuclear functions.

Nonreceptor tyrosine kinase membrane proteins undergo RIP as well. By way of example, Notch is a family of single-pass transmembrane proteins, ligands of which are usually also transmembrane proteins. Therefore, these receptors are primarily activated by cell-cell contact. This allows for cell contact–driven polarity and spatial relationship information exchange. On ligand stimulation, Notch family members, as with ErbB4, undergo sequential cleavage by tumor necrosis factor converting enzyme and γ-secretase. The intracellular domain traffics to the nucleus, where it activates, together with the *CSL* and Mastermind gene products, transcription of specific target genes (including those involved in the p53 tumor suppressor and colon carcinoma pathways, as well as those involved in myogenesis and myopathies).[8]

An imperative clinical target that is subject to the RIP process is the amyloid precursor protein (APP). The normal function of APP is still under investigation, but it is probably linked to neuronal outgrowth or maintenance.[9] Cleavage of APP by γ-secretase leads to accumulation of the hydrophobic amyloid-β peptide (Aβ) in the extracellular space, and aggregation or clustering of the peptide produces the plaques and fibrils characteristic of Alzheimer disease (AD). Several γ-secretase inhibitors have been found to significantly reduce Aβ deposition in animal models,[10] and ongoing clinical trials are directed toward inhibiting γ-secretase activity in patients. For example, BMS-708163 is now in phase II clinical testing. Phase I trials showed that it decreases cerebrospinal fluid Aβ levels by approximately 30% at a daily dose of 100 mg and by approximately 60% at a daily dose of 150 mg (28 days of treatment). It also seems to be about 190-fold more selective for APP than Notch, suggesting that it may have reduced adverse effects. Despite

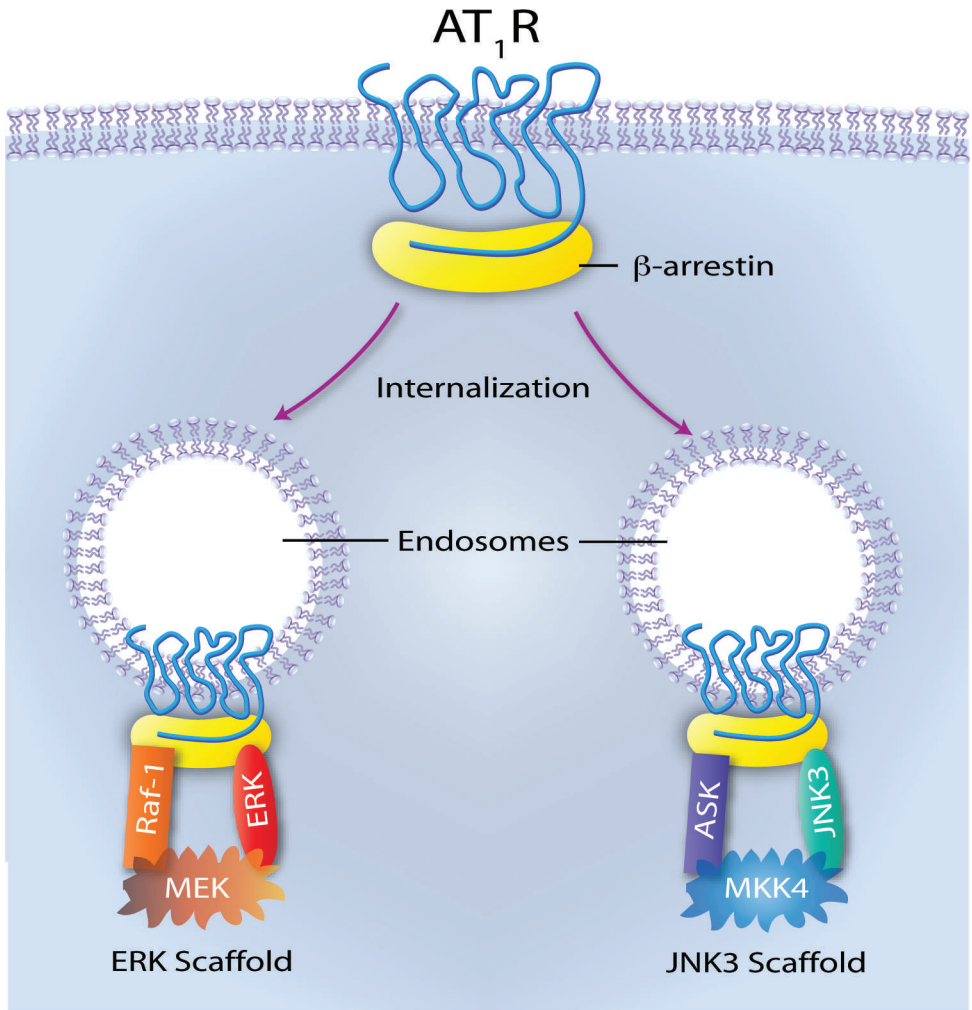

Figure 2. GPCRs can continue to signal following internalization. Following arrestin-mediated internalization, the AT_1R can continue to signal through both ERK and JNK3 scaffolds, which permit phosphorylation activation of proteins downstream in the signaling pathways.

these encouraging results, the disease seems to be complex, and reduction of the Aβ fragment alone may not be sufficient to rescue patients. The amyloid intracellular domain, which is generated simultaneously with the Aβ peptide by the γ-secretase complex, may also contribute significantly to the disease pathogenesis.[11] Mouse studies have shown that a single point mutation in the amyloid intracellular domain (D664A), which prevents intracellular domain formation, is sufficient to rescue mice from an AD phenotype, despite a high load of Aβ deposits and significant plaque formation. In these mutant mice, synaptic loss, astrogliosis, neural atrophy, and behavioral abnormalities were completely prevented. Therefore, accumulation of the intracellular fragment may also contribute to disease progression.[11]

It is estimated by the Alzheimer Research Forum (http://www.alzforum.org/dis/tre/drc/detail.asp?id=124) that 5.3 million Americans are living with AD, with a new development occurring every 70 seconds; AD is the seventh leading cause of death in the United States. A better understanding of the cellular biology and biochemistry of the RIP of APP will be needed to develop the most effective AD prevention drugs; any anticipation of a cure depends on a detailed understanding of the molecular pathogenesis of the disease.

In addition to these single-pass transmembrane receptors, several GPCRs, including V2, β_2-adrenergic receptor, and endothelin B, are reported to undergo regulated limited proteolysis to produce peptides with possible bioactivity. However, for most GPCRs, it is unclear whether an intracellular fragment (compared with an ectodomain fragment) is also generated during proteolysis, generally because the appropriate assays have not yet been performed. In addition, several GPCRs have been identified that are associated with cellular nuclei, including those for acetylcholine, Ang II, apelin, dynorphin B, endothelin

1, and prostaglandin E_2, often using multiple approaches.[12,13] For instance, the AT_1R has been localized to nuclei by several different independent studies[13–16] using techniques that include radioligand binding and chromatin solubilization assays of rat liver nuclei, immunohistochemistry of rat brain, electrophysiology assays of rat cardiac myocytes, Ang II microinjection and calcium assays, immunocytochemistry, Western blot of rat brain neurons, and immunocytochemistry and Western blot of human vascular smooth muscle cells.[13–16] In these nuclear association studies, assays have not generally been designed to differentiate between cleaved receptor fragments and holoreceptors.

Our recent investigations specifically address the nature of the intranuclear AT_1R.[14] We genetically expressed the AT_1R as a double-fusion protein with cyan fluorescent protein fused upstream and yellow fluorescent protein fused downstream (Figure 3) in several cell types and, using deconvolution fluorescent imaging and immunoblotting (with AT_1R amino terminus– and carboxy terminus–specific antibodies), demonstrated that the AT_1R is cleaved in a ligand-dependent fashion. Little cleavage occurs in the absence of ligand; hence, the yellow and blue fluors colocalize, creating an aqua color (Figure 4A). Ang II treatment is accompanied by rapid removal of the amino terminus at the cell surface, while the carboxy terminus domain accumulates in the cytoplasm and nucleus (Figure 4B). Yellow fluorescence accumulates in the nucleus; cyan fluorescence is lost at the cell surface (this is observed as a reduction in blue fluorescence at the cell perimeter). This is consistent with the idea that a population of the AT_1R undergoes cleavage at the plasma membrane, releasing the extracellular and intracellular domains. The intracellular domain accumulates in cytoplasm and cell nuclei. We corroborated the processing events using alternate tags (short amino acid sequences, Flag upstream, and myc downstream) (data not shown). Using immunoblotting and specific inhibitors, we further confirmed that the cleavage occurs in native protein as well as in genetically tagged proteins, releasing a stable 8-kD protein within cells.[14] Investigations are under way to determine the function or effects of the AT_1R intracellular domain.

An intracellular fragment is also produced from the GPCR Dfrizzled2, a postsynaptic protein that interacts with the presynaptic protein ''wingless.'' Following endosome internalization, the intracellular domain is cleaved and translocated to the nucleus, where it is involved in transcriptional events that support synapse development.[17] Collectively, the results of these studies indicate that cleavage of receptors and other cell surface proteins, as well as accumulation of

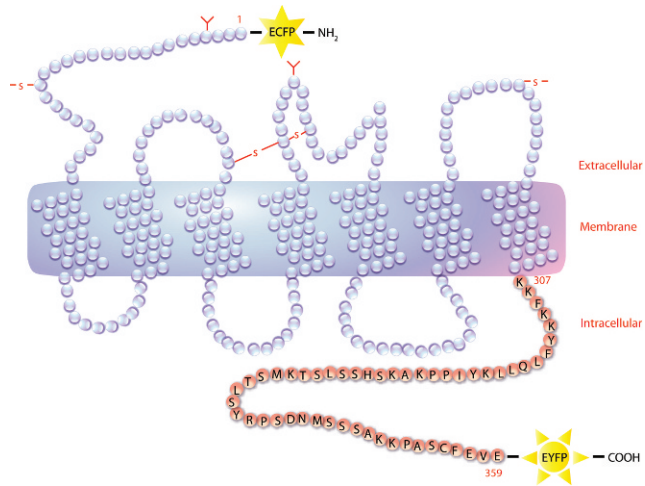

Figure 3. Fusion protein of the AT_1R. The rat AT_1R is fused in frame to upstream CFP and to downstream YFP. Fluorescent labels permit tracking and localization of proteins by fluorescent image digital capture.

stable intracellular products, can be regulated processes that serve perhaps to further amplify or enhance effects of ligand-receptor signal transduction events initiating at the plasma membrane.[5–17]

NUCLEAR MEMBRANE–ASSOCIATED RECEPTORS

In addition to downstream cellular effects of fragments RIPed from cell surface receptors, it is clear that some prototypical receptors, including GPCRs, exist as holoproteins in the nuclear membrane and possess nuclear functions. The type I lysophosphatidic acid GPCR (LPA_1) associated with hepatocytes and endothelial cells has been found in nuclear and plasma membrane cell fractions.[12] Isolated nuclei respond to LPA with increased calcium accumulation and induction of inducible nitric oxide synthase, both of which are prevented by inhibitors of LPA_1. The LPA treatment of endothelial cells also induces LPA_1 nuclear translocation and upregulates inducible nitric oxide synthase and cyclooxygenase 2.[13] Many plasma membrane receptors can be found within the nuclear membrane, including the AT_1R (Figure 4B). Because the nuclear double membrane is continuous with the endoplasmic reticulum, receptors can flow freely between the 2 compartments. The diffusion-retention model for nuclear trafficking predicts that transmembrane or integral membrane proteins in the endoplasmic reticulum can diffuse laterally in a retrograde direction from the endoplasmic reticulum to the outer nuclear membrane and then through the phospholipid bilayer flanking the nuclear pores and into the inner nuclear membrane.[18] This model further predicts that proteins will only be retained in the inner nuclear membrane at significant

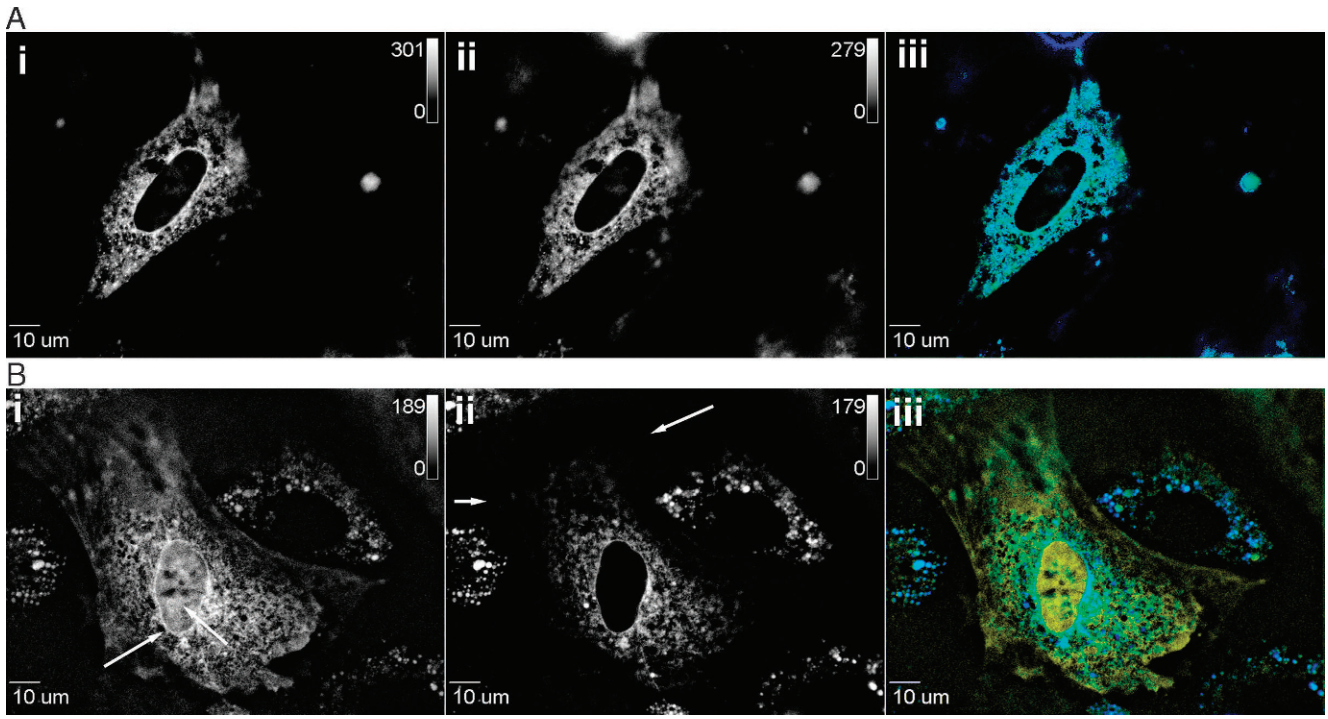

Figure 4. Cleavage of the AT_1R. Vascular smooth muscle cells were transfected with a construct encoding the AT_1R labeled at the amino terminus with CFP (blue) and at the carboxy terminus with YFP (yellow). Three-dimensional deconvolution microscopy was performed on live cells at 24 hours after transfection. A. Cells were treated with vehicle, no ligand (cleavage was not induced). (i) YFP filter image. (ii) CFP filter image. (iii) Merged images from i and ii. Colocalization of fluors produces an aqua color. B. Cells were treated with 10^{-8} mol/L of Ang II for 1 hour before imaging. (i) YFP filter image. Note the presence of YFP in the nucleus and nuclear membrane (arrows). (ii) CFP filter image. Note the loss of cyan fluorescence from the cell perimeter (arrows). (iii) Merged images from i and ii.

levels if the proteins bind to nucleosolic proteins, chromatin, nuclear matrix, or other intranuclear structures (for explanatory diagrams, see Figure 4.1 A, B in reference 4).[4] Therefore, full-length functional GPCRs like the AT_1R can accumulate in the inner nuclear membrane by retrograde trafficking from the endoplasmic reticulum. Such receptors have potential to interact with ligands present in the intranuclear membrane space and to signal events in the nucleus through nuclear membrane signal transduction events[13] that may recapitulate plasma membrane events. This represents yet another emerging area of research interest.

SUMMARY

The development of new drugs targeted to atypical intracellular receptors and receptor fragments represents a new research sphere vital to the pharmaceutical industry. GPCR-targeted drugs are generally specific for cell surface receptors and are often not specifically designed to be efficiently internalized in cells. Moreover, even those drugs that are efficiently internalized will only be effective if the original binding site (or 3-dimensional binding pocket) is intact in the internalized target membrane protein and if it is subject to ligand regulation. For example, for the AT_1R, the typical nonpeptide receptor blockers such as candesartan, losartan, valsartan, and irbesartan bind some amino acids within the agonist binding pocket that also interact with Ang II (eg, Lys^{199} in the fifth transmembrane domain and His^{256} in the sixth transmembrane domain), as well as some unique amino acids.[19–21] To the extent that these antagonists permeate the cell membrane, they could be effective in blocking the nuclear membrane–associated receptor but would likely not be effective against the cytoplasmic or nucleosolic carboxy terminus cleavage fragment. In most cases, effective targeting of cleaved fragments or intracellular domains generated from plasma membrane proteins will require novel strategies.

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