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G Protein-Coupled Receptors: Molecular Organization and Regulatory Mechanisms

Marc G. Caron, Howard Hughes Medical Institute Laboratories, Departments of Cell Biology and Medicine, Duke Univ. Med. Ctr., Durham, NC 27710 USA.

Signal transduction through G protein-coupled receptors comprises three functional components, a receptor, a G protein and a effector protein. Work over the last several years has led to the characterization of virtually all of the components of these systems. What has come out of these studies is that these mechanisms of signal transduction are pervasive in nature being found in mammalian and avian species, as well as lower organisms such as yeast and slime mold. It is known that G protein-coupled receptors mediate the action of such diverse molecules such as small hormones and neurotransmitters, small peptide molecules as well as glycoprotein hormones and various sensory perceptions such as light, olfaction and most likely taste.

The elucidation of the structure of these receptors which all appear to be comprised of seven transmembrane domains has revealed that for each hormone or neurotransmitter there is a tremendous amount of heterogeneity in the number of gene products that appear to mediate the action of a single hormone or neurotransmitter. For example, for dopamine there are now five distinct receptors as well as nine distinct adrenergic receptors with as many as 13-15 receptor subtypes that mediate the action of serotonin. The heterogeneity of components has been also observed in the case of the G proteins with as many as 17 distinct alpha subunits, four different beta subunits and as many as seven distinct gamma subunits. Whereas it is not absolutely established that every possible combination and permutation exists in various cells, it has been demonstrated that two receptors expressed in the same cell and stimulating the same signal transduction can interact with distinct beta-gamma subunits in conjunction with an alpha subunit. Therefore, some level of specificity appears to exist within single cells with respect to

even beta-gamma subunits. Characterization of the effector proteins which include adenylyl cyclases, phospholipases A and C, cyclic GMP phosphodiesterase as well as potassium and calcium channels has also revealed a tremendous amount of heterogeneity. As an example, it is now known that there are at least 9-10 different gene products that encode for the enzyme adenylyl cyclase and each of these enzymes have distinct properties with respect to their ability to interact with various alpha subunits of G protein as well as being stimulated or inhibited by beta-gamma subunits and/or stimulated by calcium calmodulin. Therefore, given this level of heterogeneity with the various components of these systems, it is not surprising that the number of possible permutations in any given cell will be rather numerous.

When one considers the properties of G protein-coupled receptors, three main properties come to mind. That is, G protein-coupled receptors bind hormones and ligands, second they activate the biochemical processes (via G protein/effectors), and third, the signaling through G protein-coupled receptors is dynamically regulated and increasing evidence indicates that covalent modification of these receptors is an important regulatory mechanism with these receptors.

Most of the work that we have performed in our group has involved catecholamine receptors. Catecholamine receptors are prototypes of G protein-coupled receptors. As mentioned already, five distinct subtypes of dopamine receptors exist whereas as many as nine distinct adrenergic receptor subtypes have been characterized. They all display the same topological organization of seven transmembrane domains. Studies of the structure-function of these receptors reveals that for small molecules such as catecholamine, the ligand binding domain resides within the transmembrane domain. Regions of the intracellular domains of these receptors especially the third cytoplasmic loop are involved in the interaction of receptors with G proteins. On the other hand, phosphorylation of serine and threonine residues within these cytoplasmic domains have been shown to mediate the uncoupling of receptors associated with agonist mediated desensitization. Two distinct aspects of these studies will be dealt with in this presentation. First, the finding that by mutagenesis we have identified the C terminal

region of the third cytoplasmic loop as being an important determinant which constrains the receptor into an inactive conformation until it can be occupied by an agonist and activated. Mutation of this region leads to constitutive activity of the receptor. Several naturally occurring mutants with constitutive activity have been recently identified as well as naturally occurring receptor subtypes that have the properties of constitutively activated receptors. Second, with respect to the mechanism of desensitization of G protein-coupled receptors, the phosphorylation of the receptors by various kinases has been shown to be important in the turn-off mechanism of the signal. Associated with this phenomenon of desensitization are two cellular trafficking events referred to as sequestration and down-regulation. We have identified a highly conserved motif in the seventh transmembrane region of the beta2 adrenergic receptor which directs the process of receptor sequestration in the agonist-mediated desensitization phenomenon.

Constitutively activated G protein-coupled receptors were originally identified by Dr. Cotecchia in our group when she found that replacing the C terminal sequence of the third intracellular loop of the alpha1 adrenergic receptor by the very similar sequence of the beta2 adrenergic receptor led to a chimeric receptor which had the ability to stimulate phosphotidylinositol turnover in the absence of agonist occupancy. It was found that single point mutations in this region also led to constitutive activation of the receptor. Thus, it was as if the mutated receptors were already in a conformation having high affinity for activation of the G protein similar to the conformation that the receptor adopts once it is occupied by an agonist. From these studies several characteristic properties of these receptors have been delineated.

(1) They generate intracellular signals in the absence of agonist occupancy.

(2) They show increased affinity for agonists.

(3) They show increased potency of agonists for the generation of intracellular signals.

(4) They often shown increased intrinsic activity of partial agonists.

(5) Classical antagonists may display negative agonist properties.

Similar mutations in the beta2 adrenergic receptor as well as the alpha2 adrenergic receptor also produce the same constitutively active phenotypic receptor.

The findings described below suggest that some naturally occurring receptor subtypes may display the intrinsic properties of constitutively active receptors. Dopamine D_{1A} and D_{1B} receptor subtypes are coupled to activation of adenylyl cyclase and exhibit a distinct anatomical localization. The primary structure of the two receptors is highly conserved within the transmembrane domains but differs within the third intracellular loops and the carboxyl tails. To identify functional differences, binding and stimulation of adenylyl cyclase were assessed in 293 and COS-7 cells expressing either D_{1A} or D_{1B} receptor. In general, membranes expressing D_{1B} receptors displayed higher affinities for agonists than those expressing D_{1A} receptors, whereas antagonists had generally a lower affinity at the D_{1B} than at the D_{1A} receptor. Basal activity of adenylyl cyclase in 293 cells expressing various levels of D_{1B} receptors was markedly higher than the basal activity measured in cells expressing D_{1A} receptors. Thus, the fold and maximal stimulation of adenylyl cyclase resulting from activation of the D_{1B} receptor was significantly less than that obtained following agonist activation of the D_{1A} receptor. These properties are reminiscent of those of constitutively active G protein-coupled receptors obtained by site-directed mutations. Experiments with chimeric D_{1A}/D_{1B} receptors reveal that part of the constitutive activity and increased affinity for agonists might also be explained by sequences of the third intracellular loop. The different anatomical distribution and biochemical properties of these D_1 receptors strengthen the functional distinctions between these two receptor subtypes. These findings represent the first appreciated example of a naturally occurring G protein-coupled receptor with the properties of a constitutively active functional protein. Recently several mutations in G protein-coupled receptors have been identified that lead to a constitutively active phenotype and represent the probable cause of certain tumors or hormone-resistance conditions.

G protein-coupled receptors undergo a dynamic regulation in response to agonist stimulation. Once a receptor is occupied with an agonist, the response to this agonist

diminishes with time despite the constant presence of the stimulus. This phenomenon which is referred to as agonist-mediated desensitization is associated with several cellular phenomena. The initial phenomenon is an uncoupling of the receptor from its ability to activate the G protein. This phenomenon is mediated by phosphorylation of the receptor by signal transduction-activated kinases such as PKA or PKC or more specifically by receptor-specific kinases referred to as G protein-coupled receptor kinases (GRK). A large family of these GRKs (six different members) has now been characterized and the mechanisms by which they specifically interact with their receptor substrates has been found to differ significantly. Whereas the ability of GRK1 (rhodopsin kinase) to interact with rhodopsin is dependent on the isoprenylation of a C terminal cystine residue, the ability of GRK2 and 3 (BARK1 and BARK2) to interact with the beta adrenergic receptor is facilitated by the interaction of the kinases with the beta-gamma subunits of G proteins. Once the receptor is phosphorylated by GRKs another protein called arrestin interacts with the receptor and abolishes the ability of the receptor to further stimulate the G protein. A family of these arresting proteins has also been elucidated.

Following the initial uncoupling of the receptor two other phenomena occur. A rapid "sequestration" of the receptor to an intracellular compartment where receptors are no longer accessible to hydrophilic ligands. At longer periods of time after agonist occupancy, the receptors are internalized and degraded (down-regulation). The role these two cellular trafficking phenomena play in the process of agonist mediated desensitization has remained obscure. In addition, the identity of the components where receptors are sequestered or desensitized has not been identified. Whether specific sequences exist on these receptors that act as internalization signal has also not been examined previously. To attempt to shed light on these issues, the studies described below were conducted.

Related members of the G protein-coupled receptor family exhibit high degrees of amino acid conservation especially in regions implicated in ligand binding and interactions with G proteins. Conversely, other sequence motifs are highly conserved across virtually all members

of this superfamily, but their role has remained obscure. One such motif, NPXXY, corresponding to amino acids 322-326 of the human β_2AR, is present at the junction of the seventh transmembrane domain and proximal part of the carboxyl terminus of virtually all G protein-coupled receptors. A similar motif, NPXY, has been implicated in the cellular recycling of proteins such as the LDL, transferrin and insulin receptors through coated pits. As mentioned above, analogous cellular trafficking phenomena referred to as sequestration (a rapid internalization of the receptors) and down-regulation (a slower loss of cellular receptors) are associated with agonist-mediated desensitization of the β_2AR. The potential role of this sequence motif in these cellular processes was assessed by replacing tyrosine residue 326 in the NPXXY sequence of the β_2AR by an alanine residue (β_2AR^{326A}) by site-directed mutagenesis. This mutation completely abolishes agonist-mediated receptor sequestration without significantly affecting the ability of the receptor to mediate activation of adenylyl cyclase, to undergo rapid desensitization and to down-regulate in response to agonist. The only significant change associated with the mutated receptor was a complete loss of its ability to resensitize following rapid desensitization. These results suggest that the processes of sequestration and down-regulation are dissociable phenomena and that the highly conserved NPXXY motif may be responsible for the ubiquitous phenomenon of agonist-mediated G protein-coupled receptor sequestration. Moreover, the lack of resensitization with the sequestration-defective mutant receptor implies that the sequestration pathway is an important mechanism by which cells re-establish the normal responsiveness of G protein-coupled receptors following removal of a stimulus.