307P INVOLVEMENT OF ACETYLCHOLINE RECEPTORS IN AUTOIMMUNE AND GENETIC DISORDERS

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The acetylcholine receptor (AChR) is one of the best studied ligand gated ion channels and is the target for both autoimmune and genetic disorders. The adult isoform of the AChR consists of α, β, δ and ε subunits; the ε replaces the γ that is present during fetal development. Each subunit has a large extracellular domain, four transmembrane domains (Ml-M4) and a cytoplasmic loop between M3 and M4 which contains phosphorylation sites. Mutations in the AChR genes have been detected in many families with congenital myasthenic syndromes. The syndromes present neonatally or later in life and result in muscle weakness, fatigue, and in some cases wasting. The functional effects of the mutations can be studied in *Xenopus* oocytes or in HEK293 cells expressing the mutant cRNAs. Mutations in the ε subunit (that defines the adult isoform) are present in >60% of cases of "AChR deficiency". In six such families that we have studied, a deletion in the cytoplasmic loop can be partially rescued by exon misplicing that leads to inclusion of intron 11 and returns the open reading frame. The "slow channel syndrome" is due to mutations that affect AChR ion channel activations, either by prolonging single openings or by prolonging bursts of normal openings. Many of these mutations are in the M2 domain that lines the pore of the AChR channel, but the primary effects of others is on the ACh binding site resulting in an increase in the duration of ACh binding. Rare mutations in the α or ε subunits lead to a "fast channel syndrome" in which the ACh-induced events are abnormally short.

The main antibody-mediated disease involving AChRs is myasthenia gravis in which antibodies lead to loss of AChR and destruction of the postsynaptic membrane. The patients become weak but can be treated by immunotherapies. The antibodies are measured by immunoprecipitation of ^{125}I-α-bungarotoxin-labelled human AChRs, and many are directed to sites on the c submits (separate from the α-BuTx-binding sites). About 15% of myasthenia gravis patients do not have detectable antibodies against the AChR. Nevertheless, application of sera from these patients to muscle cell lines leads to AChR phosphorylation and a reversible reduction in AChR function. It is proposed, therefore, that these antibodies bind to another muscle surface receptor and activate a second messenger cascade that leads to AChR phosphorylation and increased AChR desensitisation. Antibodies to AChRs in some mothers with myasthenia, and even in some healthy mothers, can inhibit the function of the fetal AChR isoform; these antibodies can cross the placenta during pregnancy and paralyse the fetus causing a severe developmental condition, known as arthrogryposis multiplex congenita, that often leads to neonatal death.

The approaches used to define the role of antibodies to AChRs in myasthenia gravis have also been used to demonstrate the presence and pathogenic role of antibodies to voltage-gated calcium channels in the Lambert Eaton myasthenic syndrome and to voltage-gated potassium channels in acquired neuromyotonia.

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308P MAPPING OF THE LIGAND BINDING DOMAIN OF THE HUMAN TACHYKININ NK2 RECEPTOR

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The tachykinin receptors belong to the rhodopsin-like GPCRs. To date three mammalian tachykinin receptor subtypes have been cloned, namely the NK1, NK2 and NK3 receptors. Their preferred physiological peptide agonists (substance P, neurokinin A (NKA) and neurokinin B) consist of a variable N-terminal region and a conserved C-terminal motif, the latter being the most essential for ligand binding.

To define this ligand-binding site more accurately, putative ligand binding residues in the extracellular and transmembrane regions were targeted for site-directed mutagenesis. Asp-79, Asn-86, Asn-90 (helix 2), Gln-109 (helix 3), Ile-202 (helix 5), His-267 Gly-273 (helix 6) and Met-297 (helix 7) appear to be important for NKA binding but not for the binding of the non-peptide antagonist SR 48968. His-198 on the other hand plays a role in the binding of both NKA and SR 48968 [2 and unpublished data from this laboratory]. To differentiate receptor sites which specifically interact with NKA from residues which influence binding site conformations, the binding and covalent attachment of Cys-containing NKA analogues to Cys-substituted receptors were assessed. These data reveal the interaction of Met-297 in helix 7 with Leu-9 in NKA.

These data also infer that the agonist and antagonist binding determinants in the NK2 receptor may vary. The affinities of two NK2 receptor peptide antagonists (GR 100679 and MEN 10207) and two non-peptide antagonists (SR 48968 and GR 149861) for nineteen NK2 receptor mutants were determined. The mutation Tyr-266-Phe (helix 6) selectively reduced the affinity for GR 149861. Using six related compounds this interaction was suggested to be a hydrogen bond between the piperidinol moiety of GR†149861 and Tyr-266 in the receptor (unpublished data from this laboratory). Ser-274 (helix 6) and Leu-292 (helix 7) also influence binding of this antagonist. Several residues implicated in NKA binding (Gln-109, His-198, Ile-202, Gly-273 and Leu-292) were found to be important for receptor interaction of the peptide antagonists.

In conclusion, our data suggest that subsets of NK2 receptor selective ligands interact with overlapping NK2 receptor binding sites. Part of the binding site has common elements with the small ligand receptors such as rhodopsin. However, peptide ligands appear to make more interactions with the receptor than their smaller non-peptidic counterparts particularly with side chains exposed at the extracellular surface.

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309P MOLECULAR ANALYSIS OF THE LIGAND BINDING SITE ON THE β_2 ADRENOCEPTOR

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The seminal mutation studies by Strader and coworkers led to the conclusion that agonist binding to human β_2-adrenoceptors is due to specific interaction with three amino acids in helical bundles III (Asp113) and V (Ser204, Ser207). Asp113 was demonstrated to be involved in binding the protonated amino functions in agonists and antagonists. The two serine residues in helix V appeared to be the anchor points for the catechol group in isoprenaline.

In our laboratory we demonstrated the involvement of Asn293 in helix VI in stereoselective agonist recognition and activation (Wieland *et al*, 1993). This residue emerged from a molecular modelling study of the human β_2-adrenoceptor as a candidate for mutagenesis studies. A receptor mutant in which Asn293 was replaced by Leu showed substantial loss of stereospecific isoprenaline binding and adenylyl cyclase stimulation.

Moreover, in a series of agonists the loss of affinity in this Asn293Leu mutant receptor was strongly correlated with the intrinsic activity of the compounds. Full agonists showed a 10-30 fold affinity loss, whereas partial agonists, such as clenbuterol, had almost the same affinity for wild-type and mutant receptors. Hence we further explored the role of this residue by synthesizing derivatives of isoprenaline and clenbuterol. We analyzed their affinity and intrinsic activity on the wild-type and the Asn293Leu mutant receptor. It appeared that one derivative of isoprenaline, but not of clenbuterol, showed a gain in affinity from the wild-type to the mutant receptor. This derivative had a methyl substituent instead of the usual hydroxy group in the aliphatic side chain of isoprenaline, compatible with the more lipophilic nature of the leucine residue. As a consequence of these findings we conclude that there is not just one agonist binding site on the β_2-adrenergic receptor.

Stereoselective recognition of antagonists was unaltered in the Asn293Leu mutant receptor, suggesting that the binding mode of antagonists is quite different. For practical reasons (availability of a radiolabelled agonist) we addressed this issue on the human 5-HT_{1A} receptor for which β-blockers have high affinity too. It was found that another asparagine residue, now in helix VII (Asn386), was responsible for the stereoselectivity of interaction. An Asn386Val mutation caused a strong decrease in *S*-propranolol's affinity, but less so for the *R*-isomer. This loss of affinity could be regained by replacement of the hydroxyl group of the ligand by a methoxy group, as was shown in a series of propranolol analogues that we had synthesised.

In conclusion, such a 'gain of function' approach through a combination of synthetic chemistry with molecular biology, may be useful to further our insight in the precise atomic events that govern ligand-receptor interactions.

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310P MUTATIONAL ANALYSIS OF THE INTERFACE BETWEEN RECEPTORS AND G PROTEINS: IMPLICATIONS FOR PHARMACOLOGICAL ANALYSIS AND LIGAND NOMENCLATURE.

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The C-terminal tail of G protein a subunits is a key region in regulating the affinity and specificity of interactions with G protein-coupled receptors (GPCRs). The G_i-family G proteins share a conserved Cys residue 4 amino acids from the C-terminus which is the site of pertussis toxin-catalysed ADP-ribosylation. This modification prevents functional communication with agonist-occupied GPCRs. To explore the importance of this residue in detail we generated mutant forms of $G_{i1}a$ in which this Cys was replaced by each of the other naturally occurring amino acids. Co-expression of these with the α_{2A}-adrenoceptor resulted in the agonist UK14304 displaying a full spectrum of capacity to active these G proteins (Bahia *et al.*, 1998). This was highly correlated with the n-octanol/H_2O partition coefficients of the amino acids (Bahia *et al.*, 1998). To explore the detailed effects of these mutations on agonist pharmacology we constructed fusion proteins between the α_{2A}-adrenoceptor and each of Gly^{351}, Cys^{351} and Ile^{351} forms of $G_{i1}\alpha$. Adrenaline was considered a full agonist and the capacity of this ligand to stimulate high affinity GTPase activity of the fusion protein measured (Jackson *et al.*, 1999). Using the fusion protein containing Gly^{351} $G_{i1}\alpha$ many other ligands functioned as partial agonists but surprisingly oxymetazoline, a well characterised, high affinity, partial agonist was without function and acted as an antagonist (Jackson *et al.*,1999). By contrast, oxymeta-zoline functioned as a weak partial agonist at the fusion protein containing $Cys^{351}G_{i1}\alpha$ and with yet higher relative intrinsic activity compared to adrenaline at the $Ile^{351}G_{i1}a$ containing fusion protein (Jackson *et al.*,1999). This was true for all partial agonists tested, and agonist ligands showed potency profiles in which EC_{50} values were greatest at $Ile^{351}G_{i1}\alpha$ and lowest at $Gly^{351}G_{i1}\alpha$.

None of these fusion proteins displayed marked constitutive activity. By contrast, in equivalent fusion proteins containing the 5HT_{1A} receptor, substantial constitutive activity, which could be inhibited by spiperone, was present in the fusions containing $Ile^{351}G_{i1}\alpha$ and $Cys^{351}G_{i1}\alpha$ but not that containing $Gly^{351}G_{i1}\alpha$(Kellett *et al.*, 1999). As such, spiperone would be defined as an inverse agonist at the first two fusion proteins but as a neutral antagonist at the one containing $Gly^{351}G_{i1}\alpha$. As these constructs differ by a single amino acid, and this is in the G protein rather than the GPCR, these results demonstrate that agonist pharmacology is defined not only by the GPCR but also by the G protein with which it interacts (Kellett *et al.*, 1999). This provides strong support for the idea that differential relative intrinsic activities may be observed for regulation of multiple effector pathways by a single GPCR if they result from activation of distinct G proteins (Berg *et al.*, 1998).

The identity of residue^{351} of $G_{i1}\alpha$ also can directly affect the affinity of interaction between a GPCR and G protein. In fusion proteins between the A_1 adenosine receptor and the three forms of $G_{i1}\alpha$ described above, such measures of a ternary complex between the fusion protein partners and a radiolabelled agonist ligand can be deduced from the rate of ligand dissociation. This is substantially more rapid for $Gly^{351}G_{i1}\alpha$ compared to $Cys^{351}G_{i1}\alpha$. Increasing GDP concentrations inhibit agonist binding to the fusion proteins. However, the EC_{50} for GDP increases with rank order $Gly^{351}G_{i1}\alpha$ >$Ile^{351}G_{i1}\alpha$ >$Cys^{351}G_{i1}\alpha$ consistent with the lowest affinity between the two protein partners in the $Gly^{351}G_{i1}\alpha$ containing fusion protein and the highest in the $Cys^{351}G_{i1}\alpha$ containing fusion protein (Waldhoer *et al.*, 1999)

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311P A NOVEL INTERFACE BETWEEN G PROTEIN-COUPLED RECEPTORS (GPCRs) AND SMALL G PROTEINS

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G protein-coupled receptors (GPCRs) can interact with multiple adapter proteins. These include one or more of the heterotrimeric G proteins (their classical signal-transducing adapters), arrestins and a variety of other proteins, often with potential linker/scaffolding roles. Recent evidence suggests that this list may now extend to the association with and regulation of certain small G proteins.

A range of GPCRs from the rhodopsin-related Group 1 were shown to activate phospholipase D (PLD) by a mechanism sensitive to brefeldin A (BFA; an inhibitor of ARF) and by a dominant negative construct of Rho A; two small G protein regulators of PLD. The PLD responses of several other Group 1 GPCRs were unaffected. BFA-sensitivity correlated closely with the presence of a conserved NPxxY motif in transmembrane domain 7 (tm 7). In a receptor with the rarer alternative DPxxY motif, mutation to restore the canonical form facilitated PLD responses and caused gain of BFA-sensitivity. Reciprocal mutation in an NPxxY receptor produced the inverse transition in properties.

Following solubilisation of membrane proteins in non-denaturing detergent conditions, a co-immunoprecipitation approach was used to address the possibility that the GPCRs and small G proteins were engaging in some form of physical complex to enable this signalling linkage. Examples of NPxxY but not DPxxY receptors (including the D318N mutant form but not the wild type GnRH receptor) appeared to associate with ARF and Rho-directed immunoprecipitates following agonist exposure,while immunoprecipitates of the M3, AT1 and 5-HT2A receptors contained ARF and Rho A immunoreactivity. This association seems likely to underlie the enhanced BFA-sensitive PLD activation by NPxxY GPCRs. It is doubtful that the critical tm7 motif is acting as a direct docking site but rather represents some form of conformational regulator. Furthermore, it is entirely possible that the interaction is not direct and that intermediary adapter and accessory proteins are involved. The relationships between GPCR docking of heterotrimeric G proteins and the apparent link to small G proteins will be a key area for future study.

A survey of other types of GPCR, including examples from Group 1 (other than the predominantly Gq/11-linked receptors investigated previously) indicated that the apparent ability to link to small G proteins may be widespread. Interestingly, several examples of Group 2 (secretin receptor-related) GPCRs also show this ability, although the tm7 helix in these receptors has a quite distinct conserved sequence.

The modulation of small G proteins by GPCRs (apparently by engaging in some form of interactive complex) seems likely to extend their influence over cellular function, both in terms of variety of signalling pathways controlled and perhaps also in terms of temporal domains of influence.

312P ROLE OF THE Ras GAP C2 DOMAIN IN THE CONVERGENCE OF HETEROTRIMERIC AND MONOMERIC G PROTEIN SIGNALLING

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$p21^{ras}$ proteins play a critical role in cellular proliferation and differentiation processes. In common with all guanine nucleotide-binding proteins, the biological activity of Ras proteins is dependent on the GTP / GDP bound state in which they reside. This in turn is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

A number of Ras GAPs have been identified and in addition to sequence similarity within their GTPase activation domains some GAPs also display sequence similarity within a non-catalytic region called the C2 domain. The C2 (conserved region 2) domain was originally identified in protein kinase C isoforms that become activated and membrane associated in response to Ca^{2+} elevation. Since C2 domains have been identified in a wide range of proteins with vastly diverse cellular signalling functions, the precise function of C2 domains remains unclear. In the case of $p120^{GAP}$, we have demonstrated that the C2 domain is important for its translocation from the cytoplasm to a membrane-associated location in response to Ca^{2+} elevation.

The role which the $p120^{GAP}$ C2 domain plays in mediating protein-protein interactions will now be considered. We have purified three proteins which form a complex with the GAP C2 domain which we have termed "p70", "p55" and "p120" to reflect their estimated molecular weights. p70 has been identified as annexin VI. This protein is a pivotal component in the complex and interacts directly with the GAP C2 domain and p55. Furthermore, we have identified p55 as Fyn, a member of the Src non-receptor tyrosine kinase family and p120 as Pyk2, a member of the focal adhesion kinase (FAK) family. The presence of the Ras exchange factor SOS has also been detected in this protein complex.

We have mapped the GAP C2 domain binding site within annexin VI and investigated the effect of intracellular Ca^{2+} elevation on the protein complex assembly. Using blocking peptide studies and cell lines which lack the expression of annexin VI, we have investigated the role that this protein complex plays in the regulation of p21 Ras activity. Hence, we discuss how the GAP C2 domain mediates a protein-protein interaction which may be critical for the Ca^{2+}-mediated regulation of Ras activity and may function as a major integration point between G protein coupled receptors and Ras dependent MAP kinase signalling cascades.