Allosteric regulation of G-protein-linked receptors

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Introduction

Allosteric effects are observed when there are cross-interactions between two binding processes which can occur simultaneously or sequentially; the binding of one ligand affects the binding of the second ligand. This is illustrated simply in Scheme 1 in which R can be a monomeric or multimeric protein and D and I, can be small molecules or proteins. In general, allosteric interactions are manifest if it is possible to form the ternary complex, DRL and

\[ K_3 > K_2 \]

(K3 > K4), there is negative co-operativity between the binding of L and D to R. Conversely, if

\[ K_1 < K_2 \]

(K1 < K3) positive co-operativity is manifest. The degree of co-operativity, \( K_2/K_1 \) (which must be the same as \( K_3/K_2 \)) is dependent on both D and L. A special case occurs when \( K_1 = K_2 \) and there is neutral co-operativity. In this instance, the equilibrium binding of D is unaffected by L although the binding kinetics of D may change. Compounds which exhibit neutral co-operativity are useful as antagonists of the co-operative effects of other ligands which bind to the same site but have

\[ K_2/K_1 \]

values not equal to 1.

Consider the case of R being a receptor and L the endogenous neurotransmitter or hormone. In conventional pharmacological terms, agonists mimic the actions of L by binding to the same site and activating the receptor. Antagonists compete with both L and other agonists, again by acting at the same site, but cannot activate the receptor.

The temporal pattern of activation of receptors by L and agonists may differ. In vivo neurotransmitter receptors respond to the stimulated and often pulsatile release of L. This means that the receptors are not continuously and maximally stimulated. Exogenous agonists, in contrast, produce a sustained stimulation which can result in

Abbreviations used: NMS, N-methylscopolamine; TM, transmembrane.

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desensitization of a maximal response. In order to minimize receptor desensitization, it is therefore necessary to produce a submaximal stimulation. This may be accomplished by using a partial agonist (an agonist which even at the highest concentration does not produce a maximum response) or by carefully controlling the levels of agonist in vivo. Because of pharmacodynamic considerations, this can often be difficult in practice. The same consideration applies in the case of the use of antagonists where a total abolition of the receptor signaling pathway may not be required.

This relative lack of control does not apply in the same way for a drug, D, acting at an allosteric site. In this case, the binding of D and its action will only be evident when L binds to the receptor. In other words, the temporal component of neurotransmission will be retained. The effects of D will be to enhance or attenuate the endogenous signaling mechanism (depending on whether \( K_{1} \) is greater than or less than \( K_{+1} \), respectively, and assuming that D\( \cdot \)R\( \cdot \)L as well as R\( \cdot \)L represents an activated receptor species). The extent of change of the signaling is limited by the co-operativity, \( K_{+2}/K_{+1} \), and thus the effect of D is to ‘tune up’ or ‘tune down’ the response. In the case of positive co-operative agents, ‘allosteric enhancers’, the effects will tend to be greater at those synapses releasing low levels of L, especially when DRL and RL are equally active species. Drugs acting at allosteric sites therefore offer a control of receptor function which is not found with competitive agonists and antagonists.

Allosteric sites are found on the multimeric ion-channel-coupled receptors. For example, a site on \( \gamma \)-aminobutyric acid type A (GABA\( \_ \)A\_3) receptors provides the basis for the therapeutic actions of benzodiazepines.

### An allosteric site on muscarinic receptors

Muscarinic acetylcholine receptors are members of the superfamily of G-protein-linked receptors. To date, five subtypes have been cloned and these are termed m1-m5 [1]. The genetically defined subtypes m1-m4 are equivalent to the pharmacologically defined subtypes M\(_1\)-M\(_4\) [2].

The existence of allosteric interactions at these receptors, first discovered in functional studies on atrial receptors [3], was confirmed in binding studies [4,5]. The drug used in these studies was gallamine, which also acts as a neuromuscular blocker at nicotinic receptors. It was possible to show that the interactions of gallamine with membrane-bound M\(_3\) receptors satisfied Scheme 1 for a variety of muscarinic ligands, L [4]. For all ligands tested, \( K_{+2} < K_{+1} \), i.e. the interactions with gallamine were negatively co-operative. However, the co-operativity was not the same for all ligands. In other words, gallamine binding had changed the structure–binding relationships of the competitive site.

Gallamine exerts a cardiac (M\(_2\)) selectivity in its actions relative to those at M\(_1\) and M\(_3\) receptors [4]. Subsequently, it has been found that the allosteric site is present on all five receptor subtypes [6].

The formation of a D\( \cdot \)R\( \cdot \)L complex can be characterized by the change in the kinetics of binding of allosteric agents. Gallamine slows down the off-rate of a tritiated antagonist, [methyl\(^{3}H\)]N-methylscopolamine ([\(^{3}H\)NMS) from muscarinic receptors in a dose-dependent manner. The extent of slowing at high concentrations is so pronounced that it is difficult to obtain equilibrium binding data. This type of off-rate assay has been used extensively to characterize allosteric ligands.

A large number of ligands affect \([^{3}H\)NMS kinetics. These include not only small molecules [7,8] but also proteins [9,10], polysaccharides [11] and peptides [12].

There is the inevitable question of whether all these compounds interact competitively at a single allosteric site, whether there is more than one allosteric site or whether there are non-specific effects. As yet, this has not been investigated in detail but a kinetic study has provided data that are compatible with gallamine competing with obidoxime, a cholinesterase reactivator, for an allosteric site [13].

Another question is whether some negatively co-operative allosteric agents might bind to the competitive site at low concentrations and to the allosteric site at higher concentrations, or whether...
apparently competitive agents are actually strongly negatively allosteric. This has not been resolved.

All the studies described above have involved negatively co-operative interactions, but positive co-operativity can also occur. The neuromuscular blocker, alcuronium, exhibits a 4-fold positive co-operativity with [3H]NMS at atrial (M2) muscarinic receptors but negative co-operativity at M1 receptors [14,15].

**Allosteric sites on other G-protein-linked receptors**

Amiloride and some of its analogues accelerate the off-rate of [3H]yohimbine from a1-adrenoceptors [16–18] and at higher concentrations enhance the off-rate of radiolabelled antagonists from D2 dopamine receptors [19]. However, no studies have yet been carried out to determine whether the kinetic results or the equilibrium data are compatible with Scheme 1.

Certain aminobenzoylthiophenes have been shown to enhance the binding of 3H-labelled agonists to A1 adenosine receptors and to slow down the kinetics of [3H]labelled agonist binding [20]. It was postulated that these compounds were binding to the allosteric site and, at somewhat higher concentrations, to the competitive site. The quantitative aspects of the interaction of one aminobenzoylthiophene PD 81,723 (2-amino-4,5-dimethylthien-3-yl[3-trifluoromethylphenyl] methanone) with the cloned human A1 receptor have been investigated [21]. The binding of four agonists, including adenosine, to the uncoupled low-affinity state of the receptor was enhanced up to 5-8-fold by PD 81,723. The experimental data, a series of 3H-labelled antagonist/agonist competition experiments in the presence of increasing concentrations of PD 81,723, could be fitted to the allosteric model in Scheme 1. There was no necessity to postulate an additional competitive action of PD 81,723. PD 81,723 also increased agonist potencies 4–10-fold in membrane or whole-cell function assays [21].

This evidence suggests that there is a well defined allosteric site on A1 receptors with PD 81,723 and related compounds acting as allosteric enhancers.

**The location of the allosteric site on muscarinic receptors**

The binding site for acetylcholine and competitive antagonists appears to be located within the seven postulated α-helical transmembrane (TM) segments found in all G-protein-linked receptors [22–24]. Labelling studies with an alkylating agonist or antagonist show that Asp-105 within the third TM segment of the m1 sequence is modified [25–27]. This residue, conserved in all G-protein-linked receptors which bind small cationic ligands, is considered to be the primary site of interaction of the positively charged nitrogen found in essentially all muscarinic ligands.

Experiments on the location of the allosteric site have concentrated on the site to which gallamine binds. As gallamine has three positive charges, it might be thought that an acidic residue could be important for its binding affinity. However, experiments on the asparagine mutants of the conserved Asp residues, 71 (second TM segment), 99 and 122 (at the outside and inside-facing ends of the third TM segment respectively) show little change in the $K_1$ and $K_2$ values for gallamine affinity relative to those of the wild-type m1 receptor [28]. An analogous study of m2 receptors also found only small changes in gallamine affinity when conserved acidic residues were mutated [29]. The evidence is therefore that an acidic residue is not part of the gallamine-binding site of all muscarinic receptor subtypes.

Other workers have attempted to exploit the differences in gallamine affinity for the different receptor subtypes and determine those regions of the receptor which are important for gallamine binding selectivity. Gallamine has a 30–50-fold higher affinity for m2 receptors than for m5 or m3 receptors. Accordingly, a series of chimeric m2/m5 and m2/m3 receptors were constructed [30].

The inclusion of a relatively small section of the seventh TM region, the sixth TM region and the whole of the connecting third extracellular loop of m2 receptors into m5 receptors or m3 receptors increased gallamine affinity ~5-fold. The exchange of other regions did not affect gallamine affinity. The authors concluded that an element of the binding selectivity of gallamine lies within this region of the receptor.

In another study, the highly acidic EDGE sequence of the second extracellular loop of the m2 sequence was replaced by the neutral sequence (LAGQ) of the m1 receptor to determine whether these acidic groups were responsible for the 15-fold m2/m1 selectivity of gallamine [29]. The LAGQ mutant did have an 8-fold lower affinity for gallamine and the authors concluded that the EDGE sequence was important for gallamine selectivity. However, the ability of the EDGE sequence to enhance gallamine binding to m1 receptors was not examined. In addition, experience in the use of
chimeric receptors in the neurokinin receptor field has pointed to conformational effects complicating the interpretation of experiments designed to test for loss of electrostatic interactions [31].

We are using site-directed mutagenesis to explore the amino acids which are important for the binding of gallamine. From the fact that impermeant highly charged ligands exert their allosteric effect rapidly in whole cells or tissues, it may be inferred that the allosteric site is extracellularly located. In addition, the binding of many allosteric ligands dramatically slows down the binding kinetics of $^3$H[NMS]. This suggests that the gallamine site may be located extracellular to the competitive site and that gallamine provides or generates a 'cap' to impede access to the competitive site. We have concentrated on attempting to establish the features of the gallamine-binding site which are common to all subtypes and have mutated 21 conserved residues in the postulated extracellular loops or loop/helix boundaries. Most of the residues are aromatic, polar or charged. The evidence suggests that the gallamine site is located close to and extracellular to the competitive site [32].

Conclusions
The presence of allosteric sites on receptors offers novel pharmacological means of modulating receptor function. It is not yet known whether an allosteric site is a general feature of all or a subset of the G-protein-linked receptor family or whether there are endogenous ligands which bind to such sites and regulate receptor function in vivo.