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COMMENTARY

ALLOSTERIC ANTAGONISTS OF THE MUSCARINIC ACETYLCHOLINE RECEPTOR

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Background

Muscarinic acetylcholine receptors belong to a superfamily of structurally related proteins which possess seven transmembrane spanning regions and couple to G-proteins [1–4]. Thus far, five distinct muscarinic receptor subtypes (designated m1–m5) are known to exist based on recent molecular cloning studies [2, 5, 6]. Earlier knowledge of muscarinic receptor heterogeneity was based on binding and functional studies using several subtype selective antagonists (e.g. pirenzepine, AF-DX 116, 4-DAMP and haxahydrosiladifenidol) [see Ref. 7 for review]. For the most part, the interaction of these selective antagonists with muscarinic receptors has been interpreted in the context of a simple competitive bimolecular reaction which obeys the law of mass action. In fact, the use of selective antagonists as a pharmacological tool to identify receptor types and subtypes is based on this premise [8]. However, the complex binding behavior exhibited by other compounds has pointed to the existence of an allosteric (secondary) binding site on the muscarinic receptor.

Allosteric interactions

An allosteric modulator is defined as a compound that interacts with a secondary binding site on a protein molecule to influence the affinity of another compound for a topographically distinct site. In the case of neurotransmitter receptors, the latter site is that to which agonists and their competitive antagonists bind (referred to as the primary binding site) [see Ref. 9 for review]. The allosteric effect was originally conceptualized by Monod *et al.* [10] to describe the phenomenon of site-to-site interactions on oligomeric proteins such as enzymes. In their model, which has been referred to as the concerted model, these interactions result from alterations in the quaternary structure of the protein via rotations of the subunits. Positive cooperativity occurs when the binding of one molecule of a ligand increases the affinity of the protein for subsequent molecules of the same or a different ligand. Conversely, negative cooperativity ensues when the binding of one molecule of a ligand decreases the affinity of the protein for subsequent molecules of the same or a different ligand. If the molecules involved are identical, the interaction is defined as homotropic; if the molecules are different, the interaction is defined as heterotropic. In the concerted model, homotropic interactions are necessarily positive while heterotropic ones can be either positive or negative. The sequential model proposed by Koshland *et al.* [11] accounts for the occurrence of negative-homotropic effects. The dynamic nature and fine-tuned regulatory mechanisms of the allosteric enzymes allow them to play a vital role in the regulation of metabolic pathways [12]. Analogous to the role played by the regulatory site on allosteric enzymes, the secondary or allosteric site on neurotransmitter receptors may serve to modulate the "primary" site which binds agonists and their competitive antagonists [13]. This phenomenon is of particular interest since "allosteric receptors" offer a unique site for therapeutic applications.

Identification of allosteric interactions

The molecular mechanism of interaction between a ligand and a receptor is identified with the help of several methods, all of which yield valuable complementary information [see Ref. 9 for a comprehensive review]. One piece of evidence for heterotropic cooperativity is obtained by utilizing displacement radioligand binding techniques. This binding assay involves the "competition" between an unlabeled ligand and a radiolabeled nonselective ligand for occupancy of the receptor. By analogy with the identification of homotropic cooperativity, a "pseudo" Hill plot, derived from the displacement data, is performed for the diagnosis of heterotropic cooperativity. When the Hill slope is equal to unity, the unlabeled ligand competes with the radiolabeled ligand for identical noninteracting binding sites in a competitive manner. In other words, these two ligands interact with the receptor in a manner which obeys the mass action law. In the case of positive heterotropic cooperativity, the allosteric ligand enhances rather than displaces radioligand binding in a concentration-dependent manner [14]. When the Hill slope is less than unity, the presence of negative heterotropic cooperativity is suggested but not conclusively. Another possible interpretation for

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such data is that the unlabeled ligand binds with different affinities to multiple noninteracting primary binding sites (i.e. the binding of pirenzepine to M_1 and M_2 receptors) [15]. However, while complete displacement would be achieved in the latter case, an allosteric agent elicits a maximal level of decrease in ligand binding, and this level is a function of the concentration of the ligand [14]. This is due to the ability of the ligand and the allosteric compound to bind simultaneously to the receptor, where each of them decreases the affinity of binding of the other [14, 16]. Therefore, a valuable diagnostic tool to differentiate between the two possibilities is to construct displacement curves at increasing ligand concentrations. Thus, while the curves are shifted in a parallel fashion in the case of multiple primary binding sites, increasing ligand concentration results in a progressive decrease in the maximal inhibition of binding effected by the allosteric modifier. This procedure is particularly useful in the case of antagonists which interact with the receptor competitively at lower concentrations and allosterically at higher concentrations, e.g. gallamine. In this case, the use of higher ligand concentrations enforces the need for using concentrations of the displacer at which the allosteric effects become evident [17].

Another protocol frequently employed to assess heterotropic cooperativity is the Schild regression [14, 18]. This plot, derived from either saturation binding or functional assays, is commonly utilized for calculating affinity constant values of competitive antagonists. In this context, the Schild plot is linear with a slope of unity and the x-intercept is an estimate of the negative log of the equilibrium dissociation constant of the antagonist. The main utility of the Schild plot is in its ability to detect cooperative interactions between an agonist and an allosteric ligand in functional assays. When the allosteric effector binds to a secondary site on the receptor to modify the affinity of an agonist at the primary site, the Schild plot appears curvilinear. However, a major drawback to this approach is its inability to differentiate between negative cooperativity and multiple noninteracting binding sites. In addition, one should be cautious in interpreting the effects of an unknown compound using either the displacement or the Schild plot protocols since an allosteric antagonist with a high degree of cooperativity would not be distinguished easily from a competitive one [14].

The method of additivity of dose-ratios is based on the premise that the blocking effects of two antagonists at a mutual binding site are cumulative [19]. Thus, the dose-ratio shift of the agonist dose–response curve resulting from the combination of two competitive antagonists will be equivalent to the sum of the individual dose-ratio shifts produced by each antagonist used alone minus 1. If, however, one of the compounds was to be allosteric, the ensuing combination dose-ratio shift would deviate from this expected theoretical relationship.

To date, dissociation kinetic studies represent the best method for detecting cooperativity [20]. This technique involves inducing *maximal* dissociation of a radiolabeled ligand which interacts competitively with the receptor from its binding sites in the absence and presence of the compound to be tested. Dissociation is effected either by infinite dilution or by the addition of a receptor-saturating concentration of a competitive antagonist, e.g. atropine in the case of muscarinic receptors. If the second ligand modifies the dissociation of the first under these conditions, this is taken as a strong evidence of the existence of cooperative interactions. In contrast, a competitive antagonist should have no effect on the rate of dissociation under these conditions.

Hence, it should be noted that no single experimental design is adequate for defining the presence of negative cooperativity. In fact, it is recommended that several different approaches be performed in tandem when assessing mechanisms of ligand–receptor interactions. Such a task may appear deceptively simple, but actually requires extreme carefulness and dedicated validation of the different experimental protocols.

Allosteric modulation of cardiac muscarinic receptors

The first indications of an allosteric site on muscarinic receptors came from the early work of Clark and Mitchelson [21] on the antagonism of muscarinic receptor-mediated negative inotropic responses in the heart. They revealed that the progressive increase in the degree of inhibition produced by increasing the concentration of gallamine, a neuromuscular junction blocker, was less than expected for a competitive antagonist, resulting in a curvilinear Schild plot. Subsequently, the allosteric nature of the interaction of gallamine in cardiac tissue has been confirmed with muscarinic receptor binding assays. By utilizing a dissociation kinetic protocol, gallamine (at concentrations ≥ 0.3 mM) was shown to slow the off-rate of the competitive antagonists [^{3}H]*N*-methylscopolamine and [^{3}H]quinuclidinyl benzilate from cardiac muscarinic receptors [16, 17, 22]. In saturation binding studies, 0.1 mM gallamine reduced [^{3}H]*N*-methylscopolamine binding affinity in a manner uncharacteristic of a competitive antagonist [16]. Ehlert [23], using a functional approach, demonstrated the ability of gallamine to allosterically antagonize muscarinic receptor-mediated inhibition of adenylate cyclase in rat heart.

Allosteric interactions at muscarinic receptors by a number of other neuromuscular junction blockers besides gallamine have been cited. For example, pancuronium, *d*-tubocurarine, alcuronium and tercuronium exhibit negative cooperativity as determined by either kinetic [24, 25] or functional [26, 27] assays. However, it is now known that the allosteric antagonists of cardiac muscarinic receptors represent a diverse class of drugs. Examples include the cardioselective antimuscarinic agents (AF-DX 116, methoctramine, himbacine) [28–30], ganglionic blockers and related compounds (hexamethonium, C_7/3′-phthalmidopropyl) [31, 32], antiarrhythmic agents (quinidine, lidocaine, bretylium, DPI 201-106) [33–36], and K^+(tetraethylammonium) [37] and Ca^{2+} (verapamil) [38–40] channel blockers. All of the aforementioned antagonists were confirmed to bind to an allosteric site, since they were found to decelerate ligand dissociation from the primary

binding site. Interestingly, the bradycardic agent alinidine actually accelerates the off-rate of the primary ligand, in this case ^{125}I-labeled quinuclidinyl benzilate [41]. Allosteric interactions with cardiac muscarinic receptors are not confined to receptor antagonists. Some agonists, such as McN-A-343 which is selective for M_1 receptors, influence ligand binding to the primary site at the cardiac M_2 receptor in an allosteric fashion [42]. Additionally, the cholinergic precursor and partial agonist choline has been reported to regulate [^{3}H]quinuclidinyl benzilate binding in an allosteric manner [43, 44].

Allosteric site on muscarinic receptors of neural and smooth muscle origin

An analogous allosteric binding site on muscarinic receptors derived from nervous tissue has been implicated. In N1E-115 neuroblastoma cells, dihydroiso-histrionicotoxin (a noncompetitive nicotinic acetylcholine receptor blocker) inhibits high-affinity [^{3}H]scopolamine binding in a manner not expected for a competitive antagonist [45]. The list of allosteric modulators in rat brain and neuronal cell clones has since grown to include gallamine [46–48], 4-aminopyridine [49], verapamil [50], secoverine [51], the pyridinium oximes [52, 53], pirenzepine [54], tetrahydroaminoacridine (THA) [55, 56] and methoctramine [57]. It is interesting to note that multiple allosteric binding sites on neuronal muscarinic receptors may exist [40, 48, 55].

In functional studies, methoctramine allosterically inhibits the actions of the muscarinic agonist carbamylcholine in ileal smooth muscle [58] and in heart [59]. Dicyclomine, adiphenine, hexahydroadiphenine and oxyphenium also exhibit allosteric antagonism at ileal muscarinic receptors, although these compounds display competitive antagonism at cardiac muscarinic receptors [60]. Nevertheless, it appears that cooperative interactions of drugs studied in both tissues are more prominent in the case of muscarinic receptors of the heart than in smooth muscle [27, 31, 61]. The list of remaining allosteric modulators at smooth muscle muscarinic receptors is not extensive but includes the cardioselective antimuscarinic agents methoctramine and AF-DX 116 [29], the M_1 selective antagonist pirenzepine [62], phencyclidine [63] and 8-(*N*, *N*-dimethylamino)octyl-3,4,5-trimethoxybenzoate [64].

Receptor subtype selectivity of gallamine and other allosteric agents

In addition to the allosteric effects of gallamine, this compound exhibits apparent selective binding to cardiac-M_2 muscarinic receptors. It has long been recognized that gallamine is a potent inhibitor of the negative inotropic and chronotropic responses to muscarinic receptor activation in guinea pig and cat heart, but displays weak or no antagonism of muscarinic responses in smooth muscle [21, 65, 66], Hence, prior to the work of Hammer *et al.* [15] with pirenzepine, the cardioselective antimuscarinic effects of gallamine provided early evidence for the existence of muscarinic receptor heterogeneity. In experiments using both radioligand binding [67] and autoradiographic [68] techniques, it has been shown that gallamine recognizes multiple muscarinic receptor conformations in certain tissues. These data have been interpreted in terms of the binding of gallamine with high and low affinities to M_2 and M_1 muscarinic receptor subtypes respectively. It should be noted that other antagonists which exhibit cooperative effects at muscarinic receptors in the heart are also cardioselective. For example, pancuronium, hemicholinium-3 and hexamethonium have all been shown to exhibit cardioselectivity as defined by functional studies [26, 32, 69], and cooperativity as determined by either kinetic [24, 32, 55, 70] or functional [27] assays. One possible interpretation of these data is that allosteric interactions of an antagonist with cardiac muscarinic receptors impart cardioselectivity. However, other allosteric muscarinic antagonists such as verapamil, phencyclidine, secoverine and quinidine do not exhibit selectivity for cardiac muscarinic receptors [30].

Multiple modes of interaction of antagonists with muscarinic receptors

A competitive binding mechanism, in contrast to an allosteric mode of interaction, has been ascribed to gallamine in earlier radioligand binding studies. Ellis and Hoss [71] have concluded that gallamine acts competitively with rat brain muscarinic receptors labeled with [^{3}H]quinuclidinyl benzilate. In accord with this interpretation, later studies demonstrated that the off-rate of bound [^{3}H]quinuclidinyl benzilate from the same preparation was not modified by the presence of high gallamine concentrations [72]. Other investigators have suggested that gallamine at low concentrations binds competitively and at high concentrations it binds allosterically to muscarinic receptors from rat brain [63] and heart [17, 22, 24]. The emerging picture from these studies is that gallamine, at low concentrations, competes for the primary binding site on the muscarinic receptor, while at higher concentrations it binds to an allosteric site to modulate the binding of ligands to the primary site. A similar binding scheme has been described for the allosteric antagonist verapamil at muscarinic receptors in the rat heart and cerebral cortex [38, 50]. Interestingly, the characteristics of the allosteric interaction of gallamine with muscarinic receptors depend to a large extent on the radioligand used to label the primary binding site on the receptor. For example, such effects are more apparent when tropate (e.g. *N*-methylscopolamine and atropine) rather than benzilate (e.g. quinuclidinyl benzilate) ligands are used [17]. Furthermore, the binding of charged quaternary amine ligands (e.g. *N*-methylscopolamine and *N*-methylquinuclidinyl benzilate) is more sensitive to allosteric modification than the binding of tertiary amine ligands (e.g. atropine and quinuclidinyl benzilate) [17].

Molecular mechanisms for allosteric interactions of muscarinic antagonists

An allosteric binding model for muscarinic receptors has been proposed by Stockton *et al.* [16]. In their model, the primary and secondary binding sites on the receptor simultaneously bind the drug

(e.g. the "classical" antagonist ligand [^{3}H]*N*-methylscopolamine) and the allosteric antagonist (e.g. gallamine), respectively, to form a ternary complex. The binding of gallamine produces a conformational change in the muscarinic receptor such that the affinity of [^{3}H]*N*-methylscopolamine is decreased, and vice versa. It should be noted that according to Stockton *et al.* [16] this particular allosteric protein (muscarinic receptor) is a monomer. The idea of the primary and allosteric binding sites being on the same monomeric protein unit was derived from receptor solubilization studies, where the allosteric effects of gallamine persisted in the solubilized receptor [16]. This finding would distinguish the muscarinic receptor from other allosteric proteins, such as enzymes and ligand-gated ionophore complexes, which are oligomeric in structure [10]. However, recent investigations have raised the possibility that the allosteric site might be on a Na^+ channel which is linked to the muscarinic receptor through a G-protein [73–75]. In these studies, the Na^+ channel activator batrachotoxin increased the binding of acetylcholine and carbamylcholine to muscarinic receptors in rat brainstem and heart. Conversely, the binding of these agonists to muscarinic receptors enhanced the binding of batrachotoxin to sodium channels. The "cross-talk" between the muscarinic receptor and the Na^+ channel was abolished by the addition of nonhydrolyzable analogues of GTP. The proposed existence of an allosterically interacting system containing three components (muscarinic receptor, G-protein and Na^+ channel) is in line with the premise that most allosteric proteins are oligomers. On the basis of these findings, it can be speculated that other ion channels linked to the muscarinic receptor may serve as an allosteric binding site. In fact, many allosteric antagonists of the muscarinic receptor are blockers of K^+ channels, e.g. gallamine [76], phencyclidine [77] and verapamil [78]. On the basis of these findings, we tested the ability of pertussis toxin, which uncouples cardiac muscarinic receptors from the K^+ channel [79, 80], to modify cooperative interactions in rat heart. Of a selected group of known allosteric modulators at cardiac muscarinic receptors (methoctramine, gallamine, verapamil and phencyclidine), pertussis toxin did not modify the cooperative effect of these agents [Lee NH and El-Fakahany EE, unpublished results].

Certain negatively charged aspartic acid residues have been found to be important for the binding of muscarinic receptor agonists and antagonists [81]. Interestingly, most allosteric muscarinic receptor antagonists possess multiple chemical groups which are either quaternary amines or tertiary amines which would be protonated at physiological pH (e.g. gallamine and methoctramine). In fact, Birdsall *et al.* [82] have reported recently that the ionization state of a chemical moiety on the receptor which is involved in the formation of a gallamine–receptor binary complex is altered when a gallamine–receptor–*N*-methylscopolamine ternary complex is formed. These results suggest a role of certain ionizable groups on the receptor protein in the binding of allosteric antagonists. Similarly, Melchiorre *et al.* [83] have proposed a model where methoctramine interacts with four acidic residues, two of which represent the allosteric binding site. Recent data from our laboratory support a role of some of the conserved aspartic acid residues on the M_1 muscarinic receptor in the interaction of allosteric antagonists. For example, mutagenesis of aspartic acid residue number 71 (which is located at the cytoplasmic end of the second transmembrane segment of the receptor) to asparagine resulted in significant modification of the magnitude of the allosteric interactions of gallamine and methoctramine with the receptor. However, the effects of this particular mutation on the two allosteric antagonists were in opposite directions; that is, the negative cooperativity of gallamine was reduced while that of methoctramine was enhanced [Lee NH and El-Fakahany EE, unpublished results]. This is supportive of the concept of diversity of the sites of interaction of the different allosteric antagonists [40, 48, 55]. In general, the recent findings which support a role of multiple negatively charged acidic residues in the allosteric modification of ligand binding at the primary site of muscarinic receptors may explain the differential sensitivity of tertiary and quaternary amine ligands to allosteric interactions [17].

Concluding remarks

It is becoming clear that there is a large group of muscarinic receptor antagonists which are capable of modifying the conformation and the function of the receptor in an allosteric fashion, and that this type of interaction is not peculiar to the prototype allosteric antagonist gallamine. The existence of such an allosteric regulatory site(s) on muscarinic receptors may be of physiological and pharmacological relevance. Recent efforts by several groups of investigators to unfold the molecular mechanisms of interaction of allosteric antagonists with muscarinic receptors should result in the near future in important information to assist in designing new muscarinic antagonists with higher selectivity. Furthermore, a low molecular weight soluble factor derived from embryonic chick heart and brain [84] and from calf thymus [85] which binds noncompetitively to muscarinic receptors has been identified. Although the identity of this factor is not known with certainty, it has been speculated that it could be a small peptide of six amino acid residues or less with a molecular weight of $\leq$700 daltons [84]. Elucidation of the molecular mechanisms of interaction of this factor with muscarinic receptors and the identification of other endogenous agents which interact allosterically with the receptors would be of great importance. The discovery of such allosteric modulators should provide useful insights into possible mechanisms of regulation of muscarinic receptors which are operative *in vivo*, whether under normal circumstances or in disease states which affect cholinergic function.

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