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MINIREVIEW

Allostery and Cooperativity in the Interaction of Drugs with Ionic Channel Receptors

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Summary

Allostery is an essential property of many physiological mechanisms. Cooperativity together with allostery is observed in the behavior of multisubunit receptors. Here we summarize and compare several approaches to the description and analysis of allosteric phenomena with emphasis on the receptors connected to ionic channels as a model. Several simplified methods are discussed in comparison with the microscopic kinetic scheme, affinity-efficacy separation and a thermodynamic approach.

Key words

Allostery • Cooperativity • Hill coefficient • Affinity • Efficacy • Nicotinic acetylcholine receptor

Introduction

Interaction of molecules with specific binding sites induces transfer of information to the effector part of the protein, whether it be an ionic channel, G-protein coupled receptor, transport molecule or enzyme. This interaction and its molecular consequences are often referred to as allosteric. The meaning of the term "allostery" is very wide, but almost always indicates a remote interaction between one part of a molecular complex and another. For molecules with more than one binding site for ligand allosteric transition could be considered as a transfer of information from one occupied binding site to another, which could be modified by changing its intrinsic affinity. Allosteric is also the transfer from a binding site to an effector, e.g. an ionic channel gate, G-protein binding site for GTP or enzyme catalytic domain. The allosteric character of the ligand-binding site interaction is also tightly connected to the affinity vs. efficacy problem, i.e. an effort to distinguish ligand(s) binding events from all subsequent conformational changes. Cooperativity is usually regarded as a special case of allosteric interaction between binding sites. Allostery and cooperativity are often diagnosed according to the dose-response or binding curves, i.e from the behavior of a large population of individual receptors. However, the quantification of allostery and cooperativity strongly depends on the knowledge of detailed molecular mechanisms.

Models of allostery

There are two different concepts how to describe the ligand binding and allostery as transfer of information about ligand binding from one protein site to another.

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Basic aspects of allostery could be demonstrated for the sake of simplicity on examples of receptors with only one binding site. The classical Del Castillo-Katz mechanism (Del Castillo and Katz 1957) is represented as a linear scheme (Fig. 1A) and assumes that ligand always binds to the receptor in the resting state R and the transition to active AR^* state is a result of the isomerization reaction which *follows* binding of the agonist. The isomerization is a conformation change by which the information about agonist binding is transferred from the occupied binding site to other sites of the protein. If the two ligand binding site receptor is considered, isomerization could cause not only receptor activation but also an allosteric change of affinity of the other binding site to the ligand as will be discussed later. The general scheme of ligand-receptor interaction could contain even more states, more binding sites and ligands. A common feature of these schemes is that the conformation change *follows* ligand binding. This is known as conformational induction (Bruns 1996).

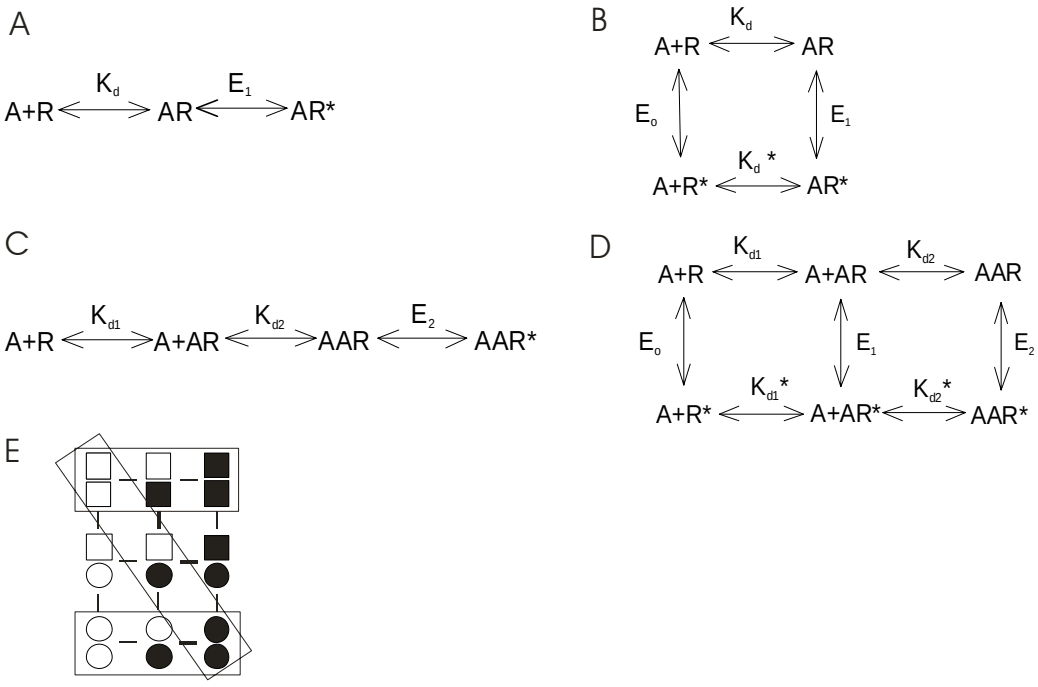

Fig. 1. Schemes showing different simplified models of allostery. **A** and **C** are del Castillo and Katz models for one and two binding sites, respectively. **B** and **D** are preformed equilibrium models for one and two binding sites, respectively. K_d are equilibrium dissociation constants for ligand binding. E_0, E_1 and E_2 are equilibrium constants for receptor isomerization. **E** is a general allosteric scheme summarizing symmetric MWC model (horizontal boxes) and sequential model (diagonal box) for receptor with two binding sites. Squares are resting subunits, circles are activated subunits. Open symbols indicate nonocupied binding sites while full symbols are occupied sites.

A historically older than the Del Castillo-Katz model was the concept of Wyman and Allen (Wyman and Allen 1951), who stated that the protein could undergo global conformation changes even spontaneously and that the ligand binding properties depend on conformation states and might be very different. But this idea became commonly accepted later, when Monod, Wyman and Changeux (MWC) proposed it as a mechanism of cooperative enzymes (Monod *et al.* (1965). The principal feature is illustrated in Figure 1B. The addition of state R^* corresponds to the opened but nonliganded channel or activation of nonliganded receptor. R^* is not only one more state in the scheme but the principal leap forward. It generally describes the situation where the conformation change does not follow the interaction of the binding site with the agonist and that more conformation states, at least R and R^*, could *coexist* in a ligand-free receptor population. Different affinity to the agonist of several coexisting receptor states causes ligands to bind to these receptors with a higher or lower probability. The important outcome is that the presence of the bound agonist could shift the equilibrium distribution between states in the direction of higher contribution of states stabilized by agonist binding. Different types of antagonists either do not distinguish between states or prefer an inactive state. This concept is known as

preformed equilibrium or *conformational selection* (Bruns 1996). Existence of the receptor in the active state in the absence of ligand can be demonstrated by constitutive activity of receptors and the occurrence of spontaneous openings of chemically activated channels without any agonist.

Allostery in receptors with more binding sites

In receptors with more binding sites, the situation is similar to enzymes with more catalytic and regulatory sites. The binding sites and ligands could be identical or there could be more types of binding sites as well as of ligands, and these sites could be activatory or modulatory. Ligands acting on activatory sites are agonists, partial agonists, competitive inhibitors or inverse agonists. Ligands acting on modulatory sites are positive or negative allosteric modulators or noncompetitive blockers.

The Del Castillo and Katz scheme with two agonist binding sites (Fig. 1C) is often used to describe simplified behavior of chemically activated channels (Ogden and Colquhoun 1983, 1985, Colquhoun and Sakmann 1985, Vyklický *et al.* 1988). In such models, allostery could be represented in two different ways. The first concerns the necessity of two agonists to be bound for effective channel opening. The second is the allosteric interaction between binding sites, which is manifested as a change of affinity of one binding site while the other binding site is occupied by the agonist or modulator.

According to the original formulation of the *symmetric* variant of the preformed equilibrium scheme with more binding sites (Figs 1D and 1E, horizontal boxes) (Monod *et al.* 1965) the behavior of binding sites is "concerted" even in the absence of the agonist. The activation of all binding sites is coordinated and symmetrical. In this model, the demands on allosteric systems seem to be too strict and the term allosteric was limited only to oligomeric proteins composed of identical subunits in equivalent positions. The idea of concerted transition and absolute cooperativity (Monod *et al.* 1965) was criticized as nonrealistic (Koshland *et al.* 1966, Weber 1975, Jackson 2002) and was soon replaced by the *sequential* model (Fig. 1E diagonal box) and other models operating with more detailed description of interaction between subunits in different conformations.

In practice, the preformed equilibrium is characterized by weak activation of nonliganded and single liganded receptors or channels as was observed in nicotinic receptors (Jackson 1984, 1989, Jackson *et al.* 1990) and NMDA receptors (Tureček *et al.* 1997).

Another physiologically important allosteric mechanism involves receptor desensitization (Katz and Thesleff 1957). Desensitized states are inactive long-living closed states of receptors with usually higher affinity to the ligand than the resting state (Cohen and Strnad 1987). Desensitized states could be involved (in Figures 1B and 1D) as one or more lines consisting of closed receptors either free or occupied by ligand(s).

Kinetic approach to receptor function

The most comprehensive information about receptor occupancy and activation is provided by understanding the complete scheme of receptor states and rate constants for transitions between them. In the kinetic formalism, the pair of kinetic constants characterizing the velocity of reversible transitions replaces all equilibrium constants in schemes shown in Figure 1. The site specific or microscopic equilibrium constants are simply a ratio of both kinetic constants characterizing transition. Ligand binding is site-specific and is described by site-specific binding and unbinding constants (Di Cera 1998) (Colquhoun 1998). The microscopic dissociation (association) constants are generally different from the global dissociation constants obtained by studying the receptor population.

The amount of information necessary to attain such a description is usually limited by the possibilities of experimental techniques. Different techniques of receptor study provide different sensitivity in time and ligand concentration. The receptor states are classified according to different criteria. The patch-clamp technique used for studying ionic channels is the most sensitive method for detecting receptor transitions at present (Hamill *et al.* 1981). It can detect the behavior of single molecules in the submillisecond time scale. Methods used in binding studies are much less accurate and hence the amount of information is more restricted. Our data about the protein states is usually indirect. Most conformational states of membrane receptors cannot be distinguished from each other in binding experiments. Moreover, the resolution of ligand binding experiments usually detects receptors in long-lasting desensitized states and not in their active state (Cohen and Strnad 1987).

The advantage of studying receptors connected to ionic channels is that electrophysiological methods can

distinguish not only the states differing in their conductance but also states differing in their lifetime. The study of statistics of duration of individual events such as opening and closing could provide additional information about kinetic constants and the number of different states.

Theoretically, it is possible for proteins to exist in an almost infinite number of different conformation states and transitions between them are in the picosecond scale. A much smaller number of states can be distinguished by their functional differences and physiological importance. In the case of ionic channels, the lifetime of such states is in the scale of 100 μs to several milliseconds. It is supposed that, from the point of view of conductance, there is only a small number of open states which do not depend on the type of agonist (Gardner *et al.* 1984, Howe *et al.* 1991). Some subconductance states could, however, be attributed to the state of channels not fully occupied by the agonist on all binding sites (for review see Karpen and Ruiz 2002)). The number of closed states could only be estimated indirectly from analysis of lifetime distributions. Even in the channel characterized by one conductance a number of closed and open kinetic states have been distinguished by their lifetimes (e.g. Hille 1992, Pallotta 1997). A very sophisticated theory has been proposed to describe and analyze kinetic transitions of ionic channels (for review see Colquhoun and Hawkes 1995, Colquhoun and Sigworth 1995). The detailed description of this approach lies outside the scope of this paper. The important result of kinetic analysis is that a „complete“ scheme of channel states has to contain tens of states to enable a description of all observed phenomena. The analysis of open and closed state lifetimes and their possible concentration dependence affords kinetic rate constants for ligand binding and unbinding from identified binding sites and rate constants for conformation changes.

In G-protein coupled receptors, which are not connected directly to ionic channels, the possibility of direct measurement of fast kinetic constants is very limited. At the present time, no methods are known for detecting rapid conversions between discrete receptor states in G-protein coupled receptors. There is a continuing discussion as to the number of such states in different receptor types. Some results obtained on G-protein coupled receptors based on a comparison of the affinities to different ligands in different states indicate that the number of states should also be larger (Kenakin 1997).

The kinetic approach affords the possibility to describe stochastic behavior of proteins in time but often this is substituted by an equilibrium or mixed approach, which is much less exact for obtaining information from the experiment. If the general kinetic scheme is known, it is easier to interpret simplified equilibrium constants even if only a part of all kinetic constants is accessible to direct measurement. The opposite approach is unfortunately generally impossible. It is very difficult to assess the structure of a model if only equilibrium constants are known and the reconstruction of kinetic constants is impossible.

A detailed description of the allosteric system is based on information about kinetic constants of every elementary step of the reaction. Allostery is described by a characteristic pattern of states, transitions between them and relationships between kinetic constants. In practice, many of these parameters are rarely measurable.

The boundary between Del Castillo-Katz and MWC models is not necessarily sharp in the kinetic description and could consist of a different frequency of events going through an open nonliganded channel and closed liganded channel in Figures 1B and 1D. Similarly, the sequential and symmetric models could be understood as extreme variants of one general scheme with a large probability of some processes and negligible probability of others.

Allostery versus cooperativity

The term allostery is used to indicate a broad spectrum of functional interactions. The relationship between allostery and cooperativity substantially depends on their definition, whether they are considered as phenomenological effect or a receptor mechanism. Kontro and Oja (1981) suppose that cooperativity in enzymology is a wider concept than allostery. They regard allostery as an action of substrates or modifiers acting from different binding sites than the catalytic binding sites causing conformational changes in proteins. This results in a change of enzyme reaction velocity. Cooperativity is related to “cooperation” of binding sites of polymeric enzymes, which is involved in a change of the classical Michaelis-Menten hyperbolic curve to a sigmoidal curve (cf. Figs 2A and 2B). The new assessment of allostery was summarized by Colquhoun (1998). Based on a detailed study of molecular mechanisms, this concept regards allostery as a more general term than cooperativity.

The beginning of analysis of allosteric

phenomena is connected with studies of hemoglobin interaction with oxygen (Hill 1910). The deviation of hemoglobin saturation curve from simple Langmuir binding isotherm was considered a manifestation of allostery. The situation of hemoglobin saturation is specific because the mechanism of oxygen binding is a physiological response by itself. In receptors connected to ionic channels or G-proteins, the effect could be separated from ligand binding event by several reaction steps and it seems essential to distinguish between binding and activation.

Allostery and cooperativity are often identified with the so-called "sigmoid kinetics" which is not generally true (Kontro and Oja 1981). The Langmuir isotherm plotted on a linear scale is hyperbolic in shape (Fig. 2A). If the binding or concentration response curves have a sigmoidal shape, this is usually accepted as a mark of allostery.

The Hill coefficient H (Hill 1910) is commonly used in biophysical studies of cooperative systems to identify allosteric interactions, although it is not an ideal quantitative measure of cooperativity. The Hill equation is an empirical formula that does not describe any known mechanism of receptor activation (Colquhoun 1998). In chemically activated channels it implies

$$I(C_X) = \frac{C_X{}^H}{C_X{}^H + EC_{50}{}^H} \quad (1)$$

where C_X is agonist concentration, $I(C_X)$ is the relative amplitude of membrane current (or generally response), EC_{50} is the apparent dissociation constant for the agonist, and H is the Hill coefficient. The Hill coefficient is usually estimated from the Hill plot (Fig. 2C) of log $(I(C_X)/(1\text{-}I(C_X))$ against $\log(C_X)$. The Hill coefficient is the slope of the line in this graph. In real cases this plot is not exactly linear and therefore the Hill coefficient varies with agonist concentration. The slope at $C_X = EC_{50}$ is usually chosen as a value indicating the degree of cooperativity. Spivak (1995) uses the value at inflexion point and Wyman (1963) suggested to use the point of median ligand activity. The Hill coefficient must be less than the number of agonist molecules that are needed to activate effectively a receptor (Kontro and Oja 1981).

Several attempts (Forsén and Linse 1995, Acerenza and Mizraji 1997, Colquhoun 1998) have been carried out to quantify cooperativity more systematically, as will be discussed later.

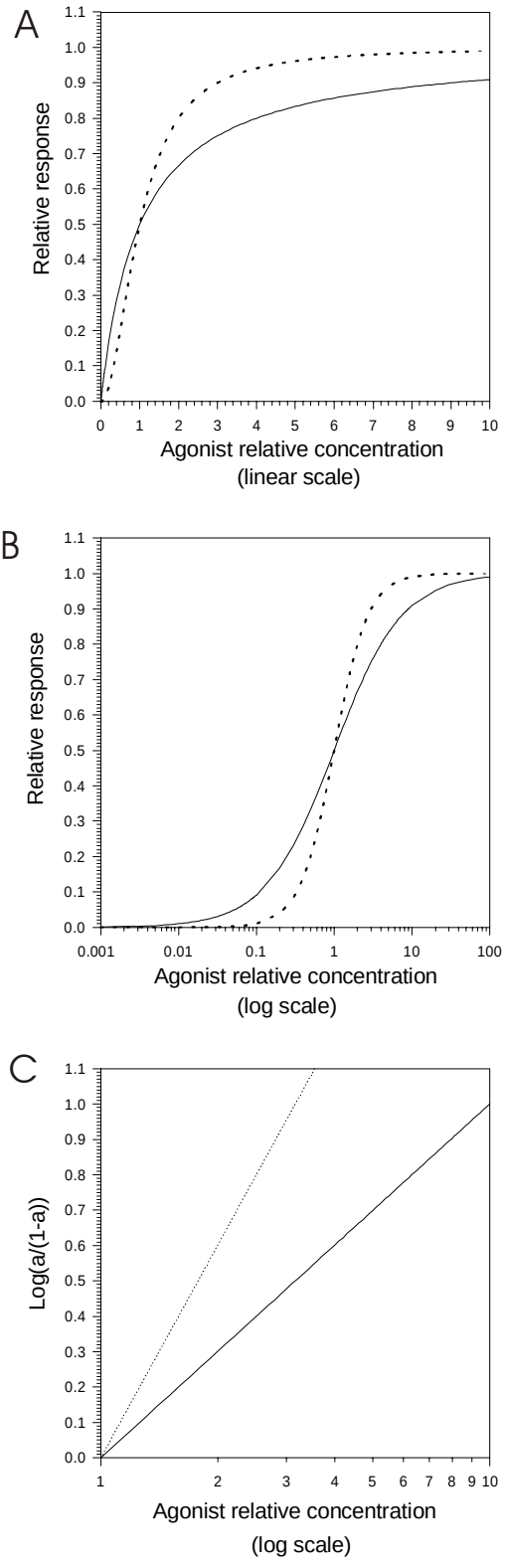

Fig. 2. Different expression of hyperbolic – non-cooperative (full line) and cooperative – sigmoidal (dotted line) binding or concentration-response curves. (**A**) Relative responses are plotted against the relative concentration. Non-cooperative kinetics is hyperbolic and cooperative kinetics is sigmoidal. (**B**) Relative responses are plotted against relative concentration on a logarithmic scale. Note that in the semilogarithmic plot, all curves are sigmoidal. (**C**) Hill plot of log (a/(1-a)) against log (a). The slope of the linear plot is proportional to the value of the Hill coefficient H.

In receptors connected to ionic channel activation, similarly to enzymes (Kontro and Oja 1981), several different mechanisms could be responsible for the sigmoidal characteristics of the concentration response curve. The first contribution to sigmoid characteristics and H>1 follows from the necessity of two binding sites to be occupied by an agonist to open the channel effectively. The presence of two activating binding sites is the general property of ligand-gated channels involved in fast synaptic transmission (Jackson 1989). The second contribution arises from allosteric coupling between different binding sites. Only the second mechanism is connected with genuine allosteric changes of affinities of different binding sites. The first mechanism is usually not regarded as cooperativity, but it is certainly allosteric because the channel gate and both binding sites are in different parts of the receptor.

Among other mechanisms contributing to the changes of apparent allostery is channel opening efficacy which is also connected with the increasing value of H (Colquhoun 1998, Krůšek and Vyskočil 2003). The difference in microscopic affinities between different binding sites contributes to the decrease of the Hill coefficient. H<1 could indicate that negative cooperativity exists (decrease of affinity of the second binding site after occupation of the first binding site) or that two different independent binding sites with different affinities participate in ligand binding. This variety of mechanisms is the cause that it is difficult to describe cooperativity by one general scheme.

The diagnostics of allostery according to the global concentration response or binding curves are problematic because their shapes are the result of several parameters. The Hill equation (1) and other kinds of simplified phenomenologic descriptions operating with a restricted number of parameters could serve only as a rough estimate. They need to be supplemented by additional information to estimate a more realistic molecular mechanism of allostery (Spivak 1995, Colquhoun 1998, Krůšek and Vyskočil 2003).

Affinity and efficacy

The analysis of ligand interaction with the receptor is strongly restricted by the possibilities of experimental techniques. The act of binding (often erroneously separated from the act of conformation change is characterized by the *affinity* of the binding site to the ligand. The following process of conformation change is characterized by the *efficacy* or intrinsic activity. It is erroneously believed that binding studies only provide information about the reaction which is connected directly to the ligand binding and unbinding whereas the functional study informs about both ligand binding and receptor activation. In the analysis of how point mutations in the receptor molecule or chemical modification of the ligand affect the final function, this misinterpretation emerges again. If the change in receptor or ligand structure influences the ligand binding, then the change should concern the functional group involved in ligand binding. This statement is not generally true. As all transitions between the receptor molecule states are parts of a complicated equilibrium, the change of equilibrium between any two states (e.g. R, R^* or AR, AR^*) could also be mediated through a chain of connected equilibria to the agonist binding step. Therefore, the binding constants obtained from the experiment are only apparent and could depend generally on all reaction steps in the scheme.

It is possible to separate the process of ligand binding and conformational changes only in the approach dealing with kinetic constants. In the so-called macroscopic approach, when simple binding or concentration-response curves are constructed, both affinity and efficacy could not simply be separated from each other (Colquhoun 1998).

Thermodynamic approach

For a better orientation in the puzzle of receptor conformation states and their interaction with ligands, it is very suitable to use the formalism of thermodynamics (Di Cera 1998, Bloomfield 2002, Jackson 2002). Thermodynamics of ligand binding and cooperativity has already been repeatedly utilized in the field of physical and polymer chemistry. The main advantage of thermodynamic state functions, such as the Gibbs free energy (G), is that their value depends only on the *present state* of the molecule but not on the pathway by which the state is attained. The absolute value of thermodynamic potentials is difficult to obtain, but for us only the changes corresponding to the conformation change or agonist binding are important as they are connected to the equilibrium constant of a given change.

$$\Delta G = RT \ln K \tag{2}$$

where K is the equilibrium constant of conformation change or equilibrium dissociation of agonist binding, R

is the gas constant and T is the absolute temperature. Results of this simple rule are very important in the studies of transition of molecules between different states. If one state could be attained by several different pathways, the sum of changes of G (and the product of equilibrium constants) between original and final states should not depend on the pathway and therefore equilibrium constants of all reaction steps are not independent. The limitation that products of equilibrium constants leading to one common state by different pathways have to be identical does not provide information about the velocity (and contribution) of different branches of the reaction. Equilibrium constants are expressed only as the ratio of kinetic constants and this does not provide any information about the absolute value of kinetic constants and therefore about the reaction velocity. The reaction velocity in different branches of the scheme shown in Figures 1B or 1D could be very different and some intermediate receptor states are practically not observed.

The change of binding site properties caused by allostery could be quantified as $\Delta(\Delta G)$, i.e. the change of ΔG of ligand binding when the binding site is occupied as the first or subsequently (Weber 1975, Forsén and Linse 1995). This value is equivalent to free energy of the interaction between binding sites.

Despite its inability to predict the kinetics, the thermodynamic approach to ligand binding could help to clarify several aspects of ligand binding and allostery. The application of ΔG to the MWC model shows that a concerted transition of subunits is not realistic because it requires an infinitely high interaction energy between subunits (Jackson 2002).

The concept of ΔG could help to extend the idea of allostery even to voltage-activated channels. ΔG in a voltage-activated channel is attributed to the movement of electric charge of the gating mechanism through a portion of the transmembrane field. Equilibrium between closed and open channel states is then influenced by membrane depolarization or hyperpolarization.

Several investigations point to a fundamental difference of thermodynamic parameters of molecular interaction of different receptors with agonists and antagonists (Borea *et al.* 2000). The change in Gibbs free energy (ΔG) could be differentiated into an enthalpic component (ΔH) and entropic component ($T\Delta S$) defined by Gibbs equation $\Delta G=\Delta H - T\Delta S$. The contribution of both components could be calculated from the temperature dependence of the equilibrium constant of a given interaction. It has been shown that when the agonist binding to a given receptor is entropy-driven, the binding of its antagonist is enthalpy-driven and *vice versa*. This "thermodynamic agonist-antagonist discrimination" was found in the glycine receptor (Ruiz-Gomez *et al.* 1989), $GABA_A$ receptor (Maksay 1994), 5-HT_3 receptor (Borea *et al.* 1996a), nicotinic receptor (Banerjee and Ganguly 1995, 1996, Borea *et al.* 1998), β-adrenoreceptors (Weiland *et al.* 1979, Molinoff *et al.* 1981), adenosine A_1 and A_{2A} receptors (Borea *et al.* 1995, 1996b). This can be explained by the fact that agonist interaction is connected with an allosteric change that is lacking in the antagonist interaction.

Alternative approach to cooperativity

Forsén and Linse (1995) proposed that a change in the affinity of the binding site given by $\Delta(\Delta G)$ could serve as a quantitative measure of allostery, but it neglects a contribution of initial differences in binding site properties. The unified view of Acerenza and Mizraji (1997) proposes another constant to be used instead of the Hill coefficient. The receptor is characterized by phenomenological "global dissociation quotient" which is equivalent to the dissociation constant, but is dependent on ligand concentration. The proposed general measure of cooperativity is a derivative of this global dissociation quotient with respect to ligand concentration irrespective of whether the change is caused by changes in agonist occupation of different binding sites or whether the change is caused by allosteric coupling of originally equal binding sites.

A very sound analysis of cooperativity phenomena by Colquhoun (1998) is based on the detailed knowledge of ionic channel kinetic schemes and models, but this analysis also concerns the G-protein coupled receptor. The basic change in describing allostery is the result of an analysis of kinetic constants of individual channel or receptor conformation transitions.

Spivak (1995) uses a detailed knowledge of channel mechanisms for treating the dissociation constant and Hill coefficient not as independent quantities but as being dependent on a simplified representation of equilibrium constants of binding, gating and allostery. The analysis of functional changes induced by mutations could in some cases provide information as to which part of channel machinery is involved in these changes (Kusama *et al.* 1994, Spivak 1995, Krůšek and Vyskočil 2003).

Allostery in chemically activated ionic channels

Actually, one of the relatively well studied allosteric proteins is the nicotinic acetylcholine receptor and several other chemically activated channels (Changeux 1990, Changeux and Edelstein 1994, Jackson 2002). Activation of the nicotinic acetylcholine receptor is used to illustrate principles of MWC theory including positive allosteric transition (Changeux *et al.* 1984, Changeux 1990, Jackson 1994) and spontaneous openings in the absence of the agonist (Jackson *et al.* 1990).

On the other hand, an example of negative allostery in chemically activated ionic channels is the NMDA receptor. Opening of the NMDA receptor channel requires simultaneous binding of glutamate and glycine. The glutamate binding site is localized on the NR2 subunit, while the glycine binding site is localized on the NR1 subunit. Although the binding sites for each agonist are localized in different subunits, the presence of one agonist influences the binding of the other.

The binding of glycine removes the NMDA or glutamate response desensitization (Mayer *et al.* 1989), but the affinity of glycine binding is reduced upon glutamate binding (Benveniste *et al.* 1990). This could be explained by negative allosteric coupling between these two binding sites localized on different subunits (Regalado *et al.* 2001). The activation of NMDA receptors is also allostericaly modulated by neurosteroids (Wu *et al.* 1990, 1991, Park-Chung *et al.* 1994, 1997, Abdrachmanova *et al.* 2001).

It is supposed that up to 20 % of nicotinic receptors could be in closed high-affinity desensitized states in the absence of the agonist (Changeux 1990). Because the kinetics of recovery from desensitized states is usually slower than receptor activation, a substantial proportion of the channel population is liganded but not open after a prolonged presence of the agonist. The physiological role of desensitized states of chemically activated channels could protect cells from high concentrations of the agonist. On the other hand, an increased affinity of desensitized nicotinic receptors is near to the nonquantal release concentration of acetylcholine in the synaptic cleft in the presence of cholinesterase inhibitors (Katz and Miledi 1977, Vyskočil and Illes 1977). Properties of the desensitized state of nicotinic receptor are subject of modulation by the microenvironment of the receptor (Magazanik and Vyskocil 1975, Magazanik *et al.* 1982, Giniatullin *et al.* 2001).

Conclusions

The study of allosteric and cooperative effects has given a deeper insight into common molecular mechanisms. Allosteric mechanisms govern the function of different types of receptors, ionic channels and enzymes. At the cellular level, allosteric proteins could be found at different levels of cellular signaling and regulation. A common description of allostery at the molecular level based on kinetics has hitherto been inaccessible directly for many proteins for methodical reasons. Despite the lack of direct evidence in receptors not connected directly to ionic channels the framework of rapid discrete transitions between conformation states is widely accepted. It is only a question of time and progress in experimental techniques when it will be possible to obtain a more reliable kinetic description of allosteric phenomena not only in ionic channels but also in other receptors.

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