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Modulatory drug action in an allosteric Markov model of ion channel behaviour: biphasic effects with access-limited binding to either a stimulatory or an inhibitory site

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Abstract

Concentration-dependent biphasic effects of drugs on ion channel activity have been reported in a variety of preparations, usually with stimulatory effects seen at low concentrations followed by increasingly dominant inhibition at higher levels. Such behaviour is often interpreted as evidence for the existence of separate modulatory drug binding sites. We demonstrate in this paper that it is possible for biphasic effects to be produced in an allosteric model of a ligand-activated ion channel, where diffusion-limited binding of the modulatory drug is restricted to either a stimulatory or an inhibitory site (but not both) because of steric overlap. The possibility of such an interaction mechanism should be kept in mind when interpreting experimental data if stoichiometric evidence from complementary techniques suggests that only one drug molecule is bound per receptor/ion channel complex.

Keywords: Non-competitive inhibitor; Nicotinic receptor; Burst behavior; Simulation and modelling; Dual action

1. Introduction

Blockade of ion channel activity by drugs is often usefully interpreted in terms of linear sequential models [1,2], with blocked and open states directly connected as shown in the following simple scheme (Scheme 1).

In this ligand-activated channel, A is the agonist molecule, D is the drug which has non-competitive inhibitory effects, the ks are association and dissociation rate constants, and β and α are the channel opening and closing rate constants, respectively, with states (shown in parentheses) 1, 2, 3 and 5 being closed, and state 4 open. In such a scheme, peak macroscopic ion channel activity within the time frame of synaptic events will decrease monotonically as the concentration of D is increased. However, in several systems reported in the literature, this relationship may be more complex, with increased channel activity seen at low concentrations followed by inhibitory effects at higher concentrations (e.g., effects of ethanol on NMDA [3] and $GABA_A$ receptors [4], butanol and hexanol on 5-HT_3 receptors [5], 5-HT on neuronal nicotinic receptors [6], 1-methylgalanthamine on PC12 nicotinic receptors [7], 3α-tropanyl-1H-indole-3-carboxylic acid ester on

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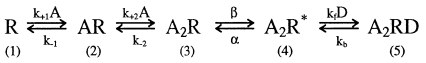


Scheme 1.

$GABA_A$ receptors [8], and endothelin-1 on Ca-activated *K* channels [9] and L-type Ca channels [10]). This type of behaviour is sometimes interpreted to mean that there are at least two sites on the channel [11]—a high affinity site which is stimulatory, and a lower affinity site (or sites) that is inhibitory and increasingly dominant at higher concentrations.

One particular system which has been extensively studied in this context is the skeletal muscle nicotinic acetylcholine receptor. It has been known for many years that a large family of non-competitive inhibitors (NCIs) can interact with this receptor [12], and analysis of the concentration and voltage dependence of block has generally led investigators to conclude that the inhibitory action is due to steric occlusion of the aqueous ion channel pore. Less commonly, discrepancies between experimental data and the predictions of this simple model have been reported (e.g., Refs. [13,14]), with many of these studies concluding that some allosteric mechanism must be involved, without quantitatively testing specific models.

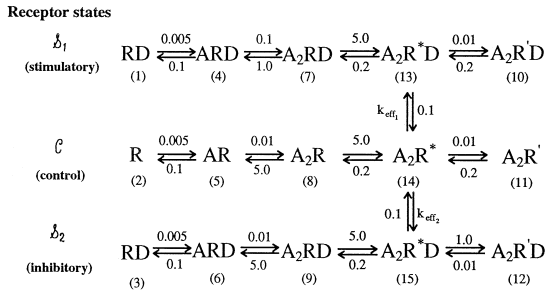
Our group has for some time been using primarily electrophysiological techniques to study the interaction of the opiate antagonist naltrexone with nicotinic receptors. Following the initial observation of biphasic modulatory effects in patch clamp records [15], further voltage clamp and radioligand binding studies were undertaken [16] which suggested that low micromolar concentrations of naltrexone enhanced the binding of acetylcholine to nicotinic receptors in addition to causing classical concentration-dependent blockade at higher levels. Detailed analysis of single channel records in a later study [17] suggested that the steric occlusion model was unlikely because state 5, a closed species shown as A_2RD in Scheme 1, was present in the absence of naltrexone. These results also supported the earlier biochemical experiments [16] indicating that the affinity of acetylcholine binding was increased. Together with evidence (i) for a single high affinity NCI binding site located in the lumen of the channel [18], (ii) that this pore is about 20–25 Å in diameter from the mouth down to the narrow gating region at the level of the plasma membrane [19], and (iii) that naltrexone is not a large enough molecule to occlude the pore if it binds at any region above the membrane level [20], we raised the question of whether the stimulatory and inhibitory effects of naltrexone could result from binding of a single drug molecule within the pore, allosterically affecting transition rates in the normal receptor activation scheme [17].

Independent support for the concept that at least some inhibitors may bind outside the narrow gating region was recently obtained in a fluorescence resonance energy transfer study [21]. In this work, it was concluded that the NCI site was 46 Å above the membrane level. Binding at this position implies that even if only inhibitory effects on channel activity are seen, they are mediated allosterically since most NCI molecules are too small for physical occlusion. Consideration of this alternative interaction mechanism naturally leads on to the possibility that other processes in nicotinic receptor channel activation and deactivation (e.g., agonist binding, channel opening and closing and desensitisation) may be affected allosterically by NCI molecules, as happens in many other systems (e.g., benzodiazepine effects on $GABA_A$ receptors, and glycine and polyamine effects on NMDA receptors). However, it is not generally known whether binding of a single modulatory drug molecule to a receptor protein could, via separate sites with varying affinities and effects on different channel transition rates, result in other than monotonic changes in channel behaviour. This question was examined theoretically in the present study by considering simple extensions to a standard Markov model of ion channel behaviour [2].

2. Theory and methods

2.1. Activation scheme

Under control conditions, we considered a five-state allosteric model alternative to Scheme 1, shown as control states $\mathscr{C}$ in Scheme 2 where A_2R^1 is a short-lived closed species present in the absence of modulatory drug *D* and directly connected to the open state A_2R^*. In the presence of *D*, parallel



Scheme 2.

activation pathways can be formed through binding of D to either of two modulatory sites S_1 and S_2 shown in Fig. 1, with connection to the control state being made through A_2R^*.

Sites 1 and 2 are separate binding sites in the pore that are sufficiently close to one another such that only a single molecule of D can be bound at any one time because of steric overlap. When D is bound to either site, there is still sufficient room within the pore for passage of ions, so that all three states labelled with an '*'(i.e., 13, 14 and 15) are conducting, with the remainder being non-conducting. Sites 1 and 2 are assumed to have stimulatory and inhibitory effects, respectively, on channel activity through allosterically-induced changes in receptor activation transition rates. Conversion of receptor from states $\mathscr{S}_1$ to $\mathscr{S}_2$ cannot occur without dissociation of D. Finally, it is assumed that binding of D to the receptor is relatively fast (i.e., forward association rate constant $k_f \geq 2 \times 10^3$ M^{-1} ms^{-1}) but at high concentrations becomes diffusion-limited in the confines of the pore as follows:

$$k_{\mathrm{eff}_i} = \frac{k_{\max} \cdot D}{K_{D_i} + D},$$

where k_{eff_i} is the effective Markov model transition rate for binding to sites S_1 and S_2, K_{D_i} is the corresponding equilibrium dissociation constant for binding of D with $k_{b_1} = k_{b_2} = 0.1$ ms^{-1}, $k_{f1} = 2 \times 10^4$ M^{-1} ms^{-1} and $k_{f_2} = 2 \times 10^3$ M^{-1} ms^{-1}, and $k_{\max}$ is a constant ($= 0.1$ ms^{-1}) such that k_{eff_i} at low drug concentrations approaches the product $k_{f_i} \cdot D$. Approximate values for the transition rates (q_{ij}, units in ms^{-1}) in Scheme 2 were suggested by experimental data at low agonist concentration [17], together with the desire to avoid unnecessary complexity where this could compromise understanding of the primary concept of biphasic behaviour.

The channel depicted in Scheme 2, with the transition rates shown, will exhibit bursting behaviour. A critical closed time (t_c) therefore had to be set for defining intra-burst closures, and this was fixed at 20 ms to be consistent with analysis of experimental results [17]. This meant that a burst of channel openings would be characterised by rapid transitions between open states (13, 14 and 15) and other short-lived closed states, provided sojourns in the closed species were all less than t_c. The total open time within a burst was used as an index of the relationship between channel activity and concentration of drug D, as this (rather than burst duration, which at high concentrations of D includes many brief closures) would determine the charge transfer through the channel and be most relevant to the magnitude of synaptic currents at a functional level.

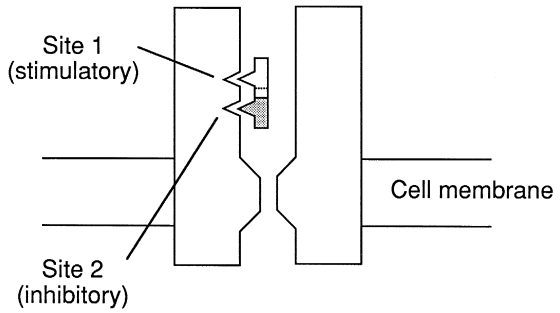


Fig. 1. Diagrammatic representation of sterically-limited binding of a drug D to two closely located but separate sites in the extracellular pore region of a nicotinic acetylcholine receptor ion channel. Only one drug molecule can bind at any one time, and movement of drug from one bound site to the other cannot occur without dissociation.

2.2. Mathematical modelling

In Scheme 1, there is a single open state (4), hence, the duration of an open sojourn has a negative exponential distribution with mean $\mu_o = 1/d_4$ where $d_4 = \alpha + k_f D$. The number of visits to the open state (N_o) during a burst has a geometric distribution, with mean $\mu_{N_o} = 1/p_o$, where p_o is the probability that a sojourn in the non-open states causes the burst to end. In some cases, this relationship can be described directly in terms of the individual parameters, and

more generally, it can be given as a matrix expression (see Appendix A, Eq. (2)). The total open time T_o during a burst is the sum of all the individual open-time sojourns; this is actually a geometric random sum of exponential random variables, and is also exponential [22] with mean $\mu_{T_o} = \mu_o \mu_{N_o} = 1/(d_4 p_o)$. Most importantly, μ_{T_o} is simply the product of the mean number of openings per burst and the mean duration of an individual open-time; this is true for any form of (scalar) random sum.

For a more complex system such as Scheme 2, where there is a class of three open states {13, 14 and 15} and a class of 12 closed states {1–12}, the arguments above need only be extended slightly for calculating appropriate means. The transition rate matrix **Q** can be partitioned according to these classes (Appendix A, Eq. (1)) and then computations done using these submatrices. The equilibrium distribution $\pi^T = (\pi_o^T \pi_c^T)$ of the process, where T denotes transposed, may be computed from **Q** (as the left eigenvector corresponding to the zero eigenvalue). Then, the mean duration μ_o of a single open-time and the mean number μ_{N_o} of openings per burst may be expressed in terms of the equilibrium distribution and the given submatrices. For Scheme 2, the product $\mu_o \mu_{N_o}$ is only an approximation of the mean of the total open-times during a burst, as N_o and the duration of an open-time are not in general independent. However, in all the numerical examples considered in this paper, the approximation is very good, and may be sufficient for many purposes. The exact result is more complicated (see Appendix A, Eq. (8)) but can be readily programmed and computed, and was in fact used for all the present examples. The number of sojourns in the closed states (all $< t_c$) during a burst is one less than the number of visits to the open states (as a burst appears to end on leaving the open states for the last time). The mean, μ_c^*, of such a conditional closed time may be computed in a similar manner to that for μ_{T_o} (see Eq. (8)). A simple and reasonable approximation to the mean duration μ_{T_B} of a burst is then $\mu_{T_B} = \mu_o \mu_{N_o} + (\mu_{N_o} - 1)\mu_c^*$. The proportion of channels in control receptor states $\mathscr{C}$ is the sum of the probabilities of being in states {2, 5, 8, 14, 11}, that is $\pi_2 + \pi_5 + \pi_8 + \pi_{14} + \pi_{11}$.

Further theoretical details are given in Appendix A. Basic Markov methods can be found in Ref. [2], together with a more complete description in Ref. [23]. All calculations in the present paper were performed using the matrix-based computer package MATLAB [24].

3. Results

Various properties of model behaviour depicted by Schemes 1 and 2 are shown in Figs. 2 and 3. Fig. 2

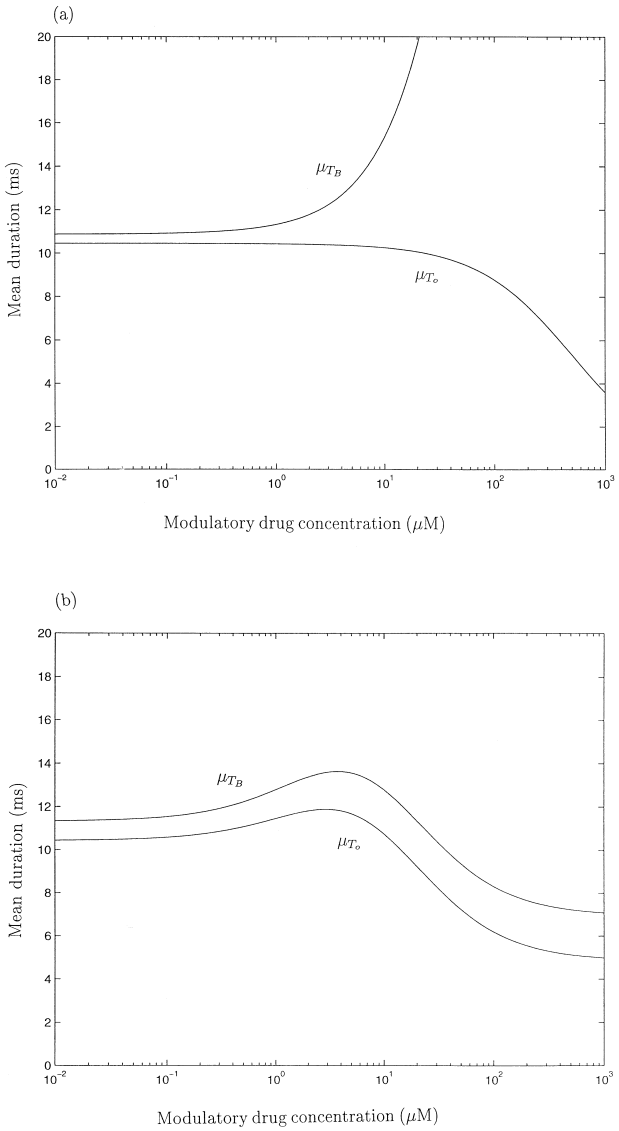


Fig. 2. Comparison of burst behaviour in the sequential and allosteric models: (a) Scheme 1 and (b) Scheme 2. The critical closed time (t_c) for defining intra-burst closures was set at 20 ms. In Scheme 1, the various q_{ij} equalled those shown in states $\mathscr{C}$ of Scheme 2, with $k_f = 2 \times 10^4$ M^{-1} ms^{-1}.

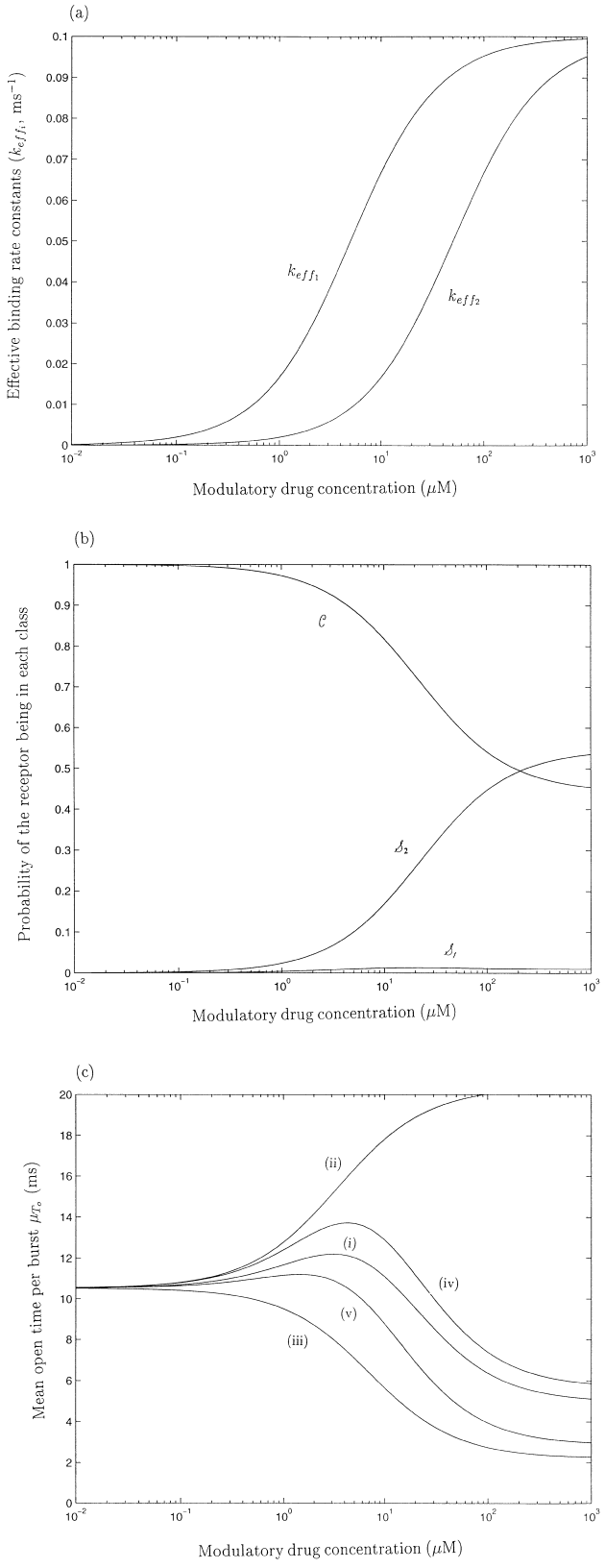


Fig. 3. (a) Diffusion-limited binding rate for drug D to sites S_1 and S_2 in the channel pore. (b) Relative occupancy of the receptor in the three classes of states $\mathscr{C}$, $\mathscr{S}_1$ and $\mathscr{S}_2$ as a function of modulatory drug concentration. (c) Effect of quantitative changes in transition rates (q_{ij}) on μ_{T_o} compared to the control values given in Scheme 2: curve (i) shows the behaviour of Scheme 2 as defined; in curve (ii), k_{f_1} has been set to zero so that binding occurs only to site S_1; curve (iii) shows the case for $k_{f_1} = 0$; curve (iv) illustrates increased stimulatory effects with $q_{4,7} = 1$ ms^{-1}; and curve (v) shows increased inhibitory effects for $q_{15,12} = 2.5$ ms^{-1}.

shows how drug D differentially affects mean burst duration (μ_{T_B}) and mean total open time per burst (μ_{T_o}). As D increases in Scheme 1 (see Fig. 2a), the number of brief sojourns in blocked state 5 increases causing μ_{T_B} to increase monotonically. In this model, μ_{T_o} would theoretically be invariant if there was no finite critical closed time (t_c) for defining intraburst closures, as bursts must effectively terminate by passing from states 4 to 3 to 2 and the transition rates for these steps (α and k_{-2}) are unaltered by D. With finite $t_c = 20$ ms, μ_{T_o} decreases at the highest levels of D because the increased number of visits to state 5 provides greater opportunity for some of these closed sojourns to exceed t_c and terminate the burst. In contrast, the behaviour of the receptor given by Scheme 2 (Fig. 2b) appears quite different when considered over a broad concentration range of D (10 nM–1000 μM). In this case, μ_{T_B} increased to a peak at about 4-μM D and thereafter declined to a plateau which was less than the original control level. The mean number of openings per burst at the peak was almost 3. A similar biphasic response was seen for μ_{T_o}, with the peak occurring at a slightly lower drug concentration (≈ 3 μM).

Fig. 3a shows an access-limited common binding rate to sites S_1 and S_2 at high drug concentration, and differential rates at low drug concentrations approximating the product $k_{f_i} \cdot D$ (K_{D_1} and $K_{D_2} = 5$ and 50 μM, respectively). The probability that the receptor will be in states $\mathscr{C}$, $\mathscr{S}_1$ or $\mathscr{S}_2$ as a function of D is shown in Fig. 3b. Occupancy of states in $\mathscr{S}_2$ is much greater than for those in $\mathscr{S}_1$ because in the former, the receptor can spend long periods in other than the gateway state 15, whereas in $\mathscr{S}_1$, the receptor is more likely to rapidly return to state 13 and thereafter move to states in $\mathscr{C}$. Fig. 3c shows the

effect on ion channel behaviour of varying particular transition rate constants in Scheme 2 compared to the default values represented by curve (i). With $k_{f_2} = 0$ in curve (ii), drug binding can only occur to site S_1, leading to monotonically increasing stimulatory effects as a function of D. Similarly, with $k_{f_1} = 0$, only inhibitory effects are seen. The relative magnitude of the stimulatory and inhibitory effects in the full model is naturally a function of how particular rate constants in states $\mathscr{S}_1$ and $\mathscr{S}_2$ vary from those in the control states $\mathscr{C}$. Increased agonist association in state $\mathscr{S}_1$ (q_{47} increased from 0.1 to 1 ms^{-1}) results in increased bursting activity and an increase in μ_{T_o}, whereas increased conversion of the open species in state $\mathscr{S}_2$ (A_2R^*D) to the longer lived closed species (A_2R^1D) produces more pronounced inhibition (curve (v)).

4. Discussion

For several years now, the sequential model of ion channel blockade has provided a useful framework for interpreting data concerning drug-induced inhibition of channel activity. While many drugs no doubt act via steric occlusion at ligand-activated channels such as the nicotinic receptor, there nevertheless seems to be a sizeable sub-family of these NCIs which exert their effects in other ways [25–27]. Often in studies directed to elucidating interaction mechanisms, it is concluded that some allosteric mechanism must operate without any quantitative testing of specific reaction schemes being undertaken. These allosteric models are admittedly more complicated than the two- and three-state linear models widely used in earlier studies [28], but with matrix methods, they can be generalised to allow the feasibility of quite complex schemes to be explored [23,29]. Several widely-available computer packages (e.g., Mathematica and MATLAB) allow the underlying calculations to be made in a straightforward manner.

The particular scenario considered in the present work was influenced by results for NCI interactions with nicotinic receptors, namely that (i) the classical linear sequential model of steric ion channel block can produce only inhibitory effects, (ii) the stoichiometry of NCI binding to the high affinity pore site is 1:1, (iii) this site may be located closer to the channel mouth than previously thought, and in this region, most NCIs would not be large enough to occlude the pore, and (iv) drug binding to the site occurs preferentially when the channel is open, with an association rate that does not increase in direct proportion to drug concentration without limit because of restricted access. In building up the model in Scheme 2, we initially considered a single pore site leading to two forms of the receptor, class $\mathscr{C}$ (control) and class $\mathscr{M}$ (modified) with the drug bound. However, as curves (ii) and (iii) in Fig. 3c illustrate, this allowed only stimulatory or inhibitory effects to be generated but not both. Biphasic effects were similarly absent when this model was generalised to provide for drug binding to both open and closed states of the receptor under conditions where microscopic reversibility prevailed. We were then led to consider the two site model of Scheme 2, which corresponds to the two overlapping sites depicted in Fig. 1 with 1:1 drug receptor stoichiometry maintained. This seemed reasonable given speculation over many years that this site may be more of a binding region than a single site, following consideration of the very broad range of chemical structures seen among NCIs [12]. The assumption that binding occurs only from the open state in Scheme 2 is undoubtedly a simplification of real events, but it has support in the literature with NCI binding often being 2–3 orders of magnitude greater when channels are in the open compared to closed states. Overall, the present results are consistent with the simple interpretation that biphasic response data can provide evidence for the existence of separate stimulatory and inhibitory sites in a receptor ion channel complex, with, however, the added possibility (not immediately intuitive) that such dual modulatory behaviour does not necessarily imply independent or joint occupancy of both sites.

The predictions for burst duration and total open time per burst shown in Fig. 2a,b appear quite different, and it might therefore be expected that distinguishing between the linear sequential model of block (Scheme 1) and an alternative allosteric model (Scheme 2) could be straightforward. Experimentally, this may not be the case since the major distinguishing features of the indices of channel activity considered here (μ_{T_B} and μ_{T_o} become apparent only at higher levels ($D \geq K_D$), and this is where additional

non-specific drug effects often cause deviations from both models. Although in the present case, experimental confirmation of significant stimulation of channel activity allows immediate rejection of Scheme 1, it is usually not a trivial task to choose an appropriate alternative allosteric model with several receptor states connected in a variety of ways, and then to determine the magnitude of associated transition rates. A further interesting feature of the allosteric model in Scheme 2 is the approach to saturation of effects seen at the highest drug levels (curve (i), Fig. 3c). This is a theoretical consequence of all such models (see Ref. [30] for a discussion of saturability in cooperative ligand–macromolecule binding phenomena), but again, can be a difficult feature to confirm in particular experimental systems because of non-specific effects.

Acknowledgements

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Appendix A

Some results on probability distributions and Markov chains are needed to obtain various properties of channel behaviour such as the total mean open-time per burst and the proportion of channels in various states. These may be obtained from some probability ideas and matrix methods. A brief outline is presented here, with further details of the general methods given in Refs. [2,23,31].

The five states in Scheme 1 may be subdivided into two classes, $O = \{4\}$ being the open state and $C = \{5, 3, 2, 1\}$ being the closed states. The continuous time homogeneous Markov chain $\{X(t);\ t \geq 0\}$ describing the process has a transition rate matrix $\mathbf{Q} = [q_{ij}]$:

$$\mathbf{Q} = \left[\begin{array}{c|cccc} -d_4 & k_f D & a & 0 & 0 \\ \hline k_b & -d_5 & 0 & 0 & 0 \\ \beta & 0 & -d_3 & k_{-2} & 0 \\ 0 & 0 & k_{+2}A & -d_2 & k_{-1} \\ 0 & 0 & 0 & k_{+1}A & -d_1 \end{array}\right] = \begin{bmatrix} \mathbf{Q}_{oo} & \mathbf{Q}_{oc} \\ \mathbf{Q}_{co} & \mathbf{Q}_{cc} \end{bmatrix}, \quad (1)$$

where $d_4 = \alpha + k_f D$, $d_5 = k_b$, $d_3 = k_{-2} + \beta$, $d_2 = k_{-1} + k_{+2} A$, $d_1 = k_{+1} A$, $\mathbf{Q}_{oo} = [-d_4]$, etc. For Scheme 2, there are three open states {13, 14 and 15} and 12 closed states {1–12}. The transition rate matrix $\mathbf{Q}$ for this scheme can be partitioned into these two classes, with the submatrices $\mathbf{Q}_{ij}$ having more entries but still the same form of partitioning of $\mathbf{Q}$. The open class now consists of more than one state.

The equilibrium or stationary distribution π for a single channel is determined by $\pi^T \mathbf{Q} = \mathbf{0}$, where T denotes transposed and $\mathbf{0}$ is a vector with all zero entries. The proportion of channels in the non-control states $\mathcal{S}_1$ and $\mathcal{S}_2$ in Scheme 2 is then $\mu_{S_1+S_2} = \pi_1 + \pi_3 + \pi_4 + \pi_6 + \pi_7 + \pi_9 + \pi_{10} + \pi_{12} + \pi_{13} + \pi_{15}$; see Fig. 3b for a plot of π_C, π_{S_1} and π_{S_2} as a function of drug concentration. The equilibrium vector π for either Scheme may also be subdivided by the open and closed classes of states, so $\pi^T = [\pi_o^T, \pi_c^T]$.

Let ϕ_o and ϕ_c be the probability distribution that a visit to the open states O (or closed states C) begins in the particular states of O (respectively, C). Then $\phi_o^T = \pi_c^T Q_{co} / \pi_c^T Q_{co} \mathbf{1}$ where $\mathbf{1}$ is a column vector with all elements unity; similarly, $\phi_c^T = \pi_o^T Q_{oc} / (\pi_o^T Q_{oc} \mathbf{1})$. These entry processes are needed to evaluate the probability p_o that a burst ends with a given visit to the closed states, and for means and other properties of the total open-time during a burst. A burst ends with a visit in C of at least the critical value t_c; this is in general:

$$p_o = \phi_c^T e^{Q_{cc} t_c} \mathbf{1}. \quad (2)$$

This expression can be written out more simply in some special cases; for example, in Scheme 1, with a burst ending only by a visit to state 2 (with $t_c = \infty$), $p_0 = p_{43} p_{32}$, where $p_{43} = \alpha / d_4$ and $p_{32} = k_{-2} / d_3$. Then the number N_o of visits to the open states during a burst has the geometric form $P(N_o = n) = (1 - p_o)^{n-1} p_o$, $n = 1, 2, \cdots$, with mean $\mu_{N_o} = 1/p_o$. In the more general case, the distribution has the geometric-like form:

$$P(N_o = n) = \phi_o^T \mathbf{R}_{t_c}^{n-1} (\mathbf{I} - \mathbf{R}_{t_c}) \mathbf{1}, \quad (3)$$

where:

$$\mathbf{R}_{t_c} = \mathbf{Q}_{oo}^{-1} \mathbf{Q}_{oc} (\mathbf{1} - e^{\mathbf{Q}_{cc} t_c}) \mathbf{Q}_{cc}^{-1} \mathbf{Q}_{co}, \quad (4)$$

and the mean number of openings per burst is:

$$\mu_{N_0} = \phi_o^T [\mathbf{I} - \mathbf{R}_{t_c}]^{-1} \mathbf{1}. \quad (5)$$

The mean open-time for a single opening for Scheme 1 is the inverse of the rate d_4 out of state 4. For Scheme 2, this becomes a matrix inverse together with the initial vector ϕ_o^T of entry probabilities, and is:

$$\mu_o = -\phi_o^T \mathbf{Q}_{oo}^{-1}\mathbf{1}. \quad (6)$$

For Scheme 1, the mean total open-time per burst is just the product $\mu_{T_o} = \mu_o \mu_{N_0} = 1/(d_4 p_o)$. For Scheme 2, the same product of Eqs. (5) and (6) is only an approximation, as there may be correlations between different events within a burst. These are usually small (correlation < 0.03 between successive open-time for all the numerical values used in Scheme 2), and the approximation appears to be generally very good. A more detailed argument yields the exact value of μ_{T_o} and also of $\mu_{T_B} = \mu_{T_o} + \mu_{T_c^*}$ as:

$$\mu_{T_o} = \phi_o^T \left[\boldsymbol{I} - \boldsymbol{R}_{t_c}\right]^{-1}\left(-\mathbf{Q}_{oo}^{-1}\right)\mathbf{1}, \quad (7)$$

$$\mu_{T_c^*} = \phi_o^T \left(\boldsymbol{I} - \boldsymbol{R}_{T_c}\right)^{-1}\left(-\mathbf{Q}_{oo}^{-1}\right)\mathbf{Q}_{oc}\left[\boldsymbol{I} - (\boldsymbol{I} - \mathbf{Q}_{cc} t_c) \times e^{\mathbf{Q}_{cc} t_c}\right]\left(-\mathbf{Q}_{cc}^{-1}\right)\mathbf{1}. \quad (8)$$

The distribution of N_o and the mean of T_o can be obtained from the joint density function $f(t_1, t_2, \cdots, t_n; n)$ of the open-times in a burst with n openings [23,31]; this is:

$$f(t_1, t_2, \ldots, t_n; n) = \phi_o^T e^{\mathbf{Q}_{oo} t_1} \mathbf{Q}_{oc}(\boldsymbol{I} - e^{\mathbf{Q}_{cc} t_c}) \mathbf{Q}_{cc}^{-1}\mathbf{Q}_{co} e^{\mathbf{Q}_{oo} t_2} \ldots \mathbf{Q}_{co} e^{\mathbf{Q}_{oo} t_n} \mathbf{Q}_{oc} e^{\mathbf{Q}_{cc} t_c}\mathbf{1}. \quad (9)$$

Integrating each of $(t_1, t_2, \cdots t_n)$ over $(0,\infty)$ yields Eq. (3), which must also have $\sum P(N_o = n) = 1$. The mean of the total open-time in a burst with n openings can be written as a sum of the means of the n separate open-times. After some simplification, Eq. (7) can be obtained.

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