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Induction of lipid-protein mismatch by xenobiotics with general membrane targets

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Many membrane-active xenobiotics, such as organic solvents or general anesthetics, are without specific target sites. A common, but as yet ill-defined physical mechanism of action is usually assumed. Displacement of 'boundary' lipid activators from functional membrane proteins by the induction of lipid/protein mismatch is now established as a candidate common physical mechanism. Multiple binding site kinetics demonstrate that xenobiotics can be strongly inhibitory at realistic membrane concentrations of below 4 mol%. A general equation for inhibition is derived.

Introduction

Biological membrane functions can be inhibited by numerous lipophilic xenobiotics. Considering only the sub-lytic concentration range, inhibitors may be highly specific and possess distinct binding sites on functional membrane proteins such as ion channels, receptor proteins or membrane enzymes. On the other hand, there are many non-specific inhibitors. The present study will deal only with these inhibitors that include organic solvents, chaotropic agents, detergents and certain drug classes such as general anesthetics. The latter are typically active at 1–4 mol% of the membrane lipid phase [1,2]. A 'unitary' mechanism of action is commonly assumed: many target sites exist and are affected by some common but as yet ill-defined physical mechanism [1,2].

Two possible 'unitary' modes of action of non-specific xenobiotic have been suggested. One mechanism is based on the fact that lipophilic xenobiotics will partition into the membrane. The induced structural changes in lipid bilayer structure (e.g., in geometry and order parameters) will then lead to functional effects such as enzyme inhibition or anesthesia [1–3]. An alternative mechanism is exemplified by the non-membraneous enzyme, luciferase, which is sensitive to a broad range of xenobiotics such as organic solvents, dyes and anesthetics [1,4–6]. In the cases of dyes [4], general anesthetics [5] and monopalmitoyllysophosphatidylcholine [6], luciferase inhibition has been shown to be due to competitive displacement of the substrate, luciferin.

The lipid dependence of functional membrane proteins leads to a possible third mode of action of lipophilic xenobiotics, namely displacement of 'boundary' lipid molecules (Fig. 1). The lipid dependence of membrane functions has been thoroughly documented [7–9]. Activation appears to involve matching and line-up processes of polar and non-polar regions of protein and lipid [7–11]. Polar group hydration and lipid viscosity are both important for function [7–9]. A displacement of protein-associated lipids has in fact been proposed for general anesthetics [1] even before the 'boundary' lipid layer was discovered by EPR spectroscopy [12]. First EPR-spectroscopic studies on the effects of general anesthetics on the 'boundary' lipids of vesicular acetylcholine receptor [13] and Ca^{2+}-ATPase preparations [14] have been reported. The exchange of 'boundary' lipid against the tested xenobiotics could be demonstrated, but no mathematical relationship between exchange and receptor or enzyme activity was established. The application of a kinetic model for lipid-dependent enzymes [15] is now reported. A new kinetic equation is presented and induction of lipid/protein mismatch is shown to lead to powerful inhibition of lipid-dependent membrane functions.

Results

Most functional membrane proteins require lipid solvation for activity. The simplest case is given by a

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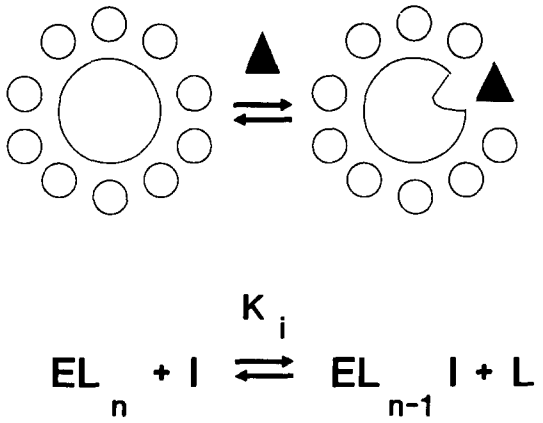


Fig. 1. Lipid/protein mismatch model. The membrane enzyme E is thought to be functionally dependent on solvation by a defined number n of lipid molecules (○). In the model considered here, displacement of one of the essential 'boundary' lipid molecule by xenobiotic (▲) or by lipid dissociation leads to complete loss of function. An illustrative binding equilibrium for inhibition is indicated. In this example, EI_n is the active fully lipid substituted enzyme and I is an inhibitor molecule. All intermediate forms of lipid and inhibitor binding are included in the comprehensive kinetic treatment given in Results.

hypothetical membrane enzyme with a number n of identical lipid solvation sites that all need to be occupied for activity. Relative velocity (v/V) is given by division of the concentration of the fully liganded enzyme species [EL_n] by the concentration of all enzyme species present ([E] + [EL_1] + [EL_2]...[EL_n]) [15]. Here, E is enzyme, L is lipid, v is actual velocity and V is maximal velocity. In the presence of a lipophilic inhibitor, I, the following relationship applies:

$$v/V = \frac{[EL_n]}{[E] + [EL_1] \ldots [EL_n] + [EI_1] + \ldots [EI_n] + [EL_aI_b] + \ldots [EL_bI_a]} \quad (1)$$

The indices a and b can assume any number between 1 and n, but their sum ($a + b$) cannot be higher than n. Each individual binding site of the enzyme is characterized by an identical microscopic lipid dissociation constant, K_l of the basic form: $K_l = [E] \cdot [L]/[EL]$. The inhibitor molecule I is assumed to bind to the same number n of lipid binding sites of the enzyme, with an identical microscopic inhibitor dissociation constant K_i at each site. K_i has the basic form: $K_i = [E] \cdot [I]/[EI]$. Statistical terms for the probability of ligand association and dissociation have to be introduced for each binding step, as previously described [15,16]. The above relationship for v/V is then further modified by rearrangement, application of binomial rules and cancellation of terms in analogy to previous studies [15,16]. This leads to

$$v/V = \left(\frac{1}{1 + [K_l(1 + [I]/K_i)/[L]]} \right)^n \quad (2)$$

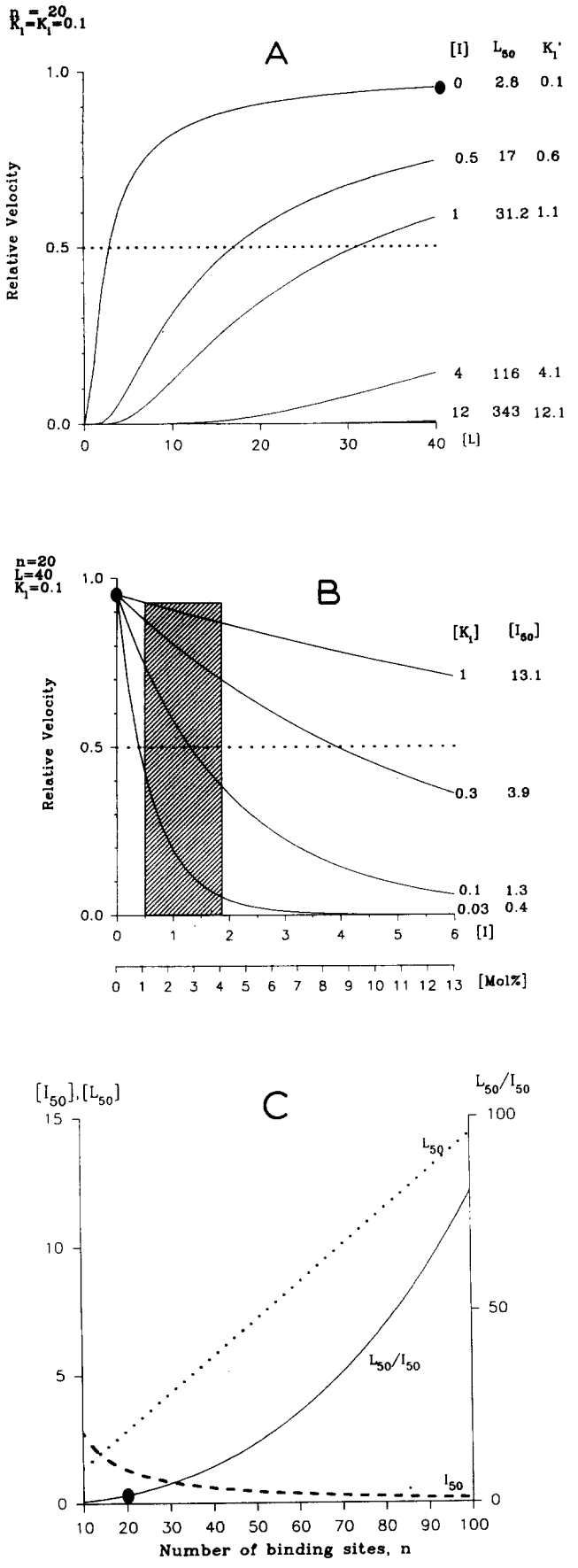
Units of molecules per protein molecule will be used here for lipid concentration, [L], and inhibitor concentration, [I], respectively. This concentration unit is particularly useful for reconstituted membrane systems as previously shown for β-hydroxybutyrate dehydrogenase, where two-dimensional phospholipid/protein dissociation binding constants could be obtained for the first time [17].

In the absence of inhibitor, Eqn. 2 converts to the simple rate equation previously derived for membrane enzymes requiring full lipid substitution (Eqn. 4 of Ref. 15). Comparison of the previous equation with the present Eqn. 1 leads to the definition of an apparent microscopic lipid/protein dissociation constant, K_l', for the presence of inhibitor, $K_l' = K_l(1 + [I]/K_i)$. This relationship is analogous to that occurring in the familiar equations for competitive enzyme inhibition by substrate displacement [18]. Deriving the cooperativity index, L_{90}/L_{10}, from the present Eqn. 2 gives the same relationship previously derived for the absence of inhibitor [15]. Inhibition by lipid displacement thus does not affect the degree of kinetic cooperativity. However, Eqn. 2 predicts a high sensitivity of lipid-dependent enzymes to low membrane concentrations of xenobiotic inhibitors. This is illustrated here for the hypothetical 'average' membrane protein previously considered [8]:

At an 1:1 mass ratio of lipid and protein and an 'average' protein molecular mass of 32 kDa, there is a total number of 40 membrane lipid molecules per protein molecule. It is the intramembraneous protein part which is important in lipid-protein interactions of the type considered. A 32-kDa membrane protein is known to have about 20 'boundary' lipid solvation sites [19]. Insertion of modelling values of $n = 20$, $K_l = 0.1$, [I] = 0 and of [L] = up to 40 lipid molecules per protein molecule into Eqn. 2 leads to a slightly sigmoidal activation curve (Fig. 2A, top curve). In the presence of various fixed amounts of an inhibitor having the same affinity as the lipid (i.e., $K_i = K_l = 0.1$), strong inhibition is obtained from Eqn. 2 (Fig. 2A, lower curves). The lipid concentrations required for half-maximal activation (L_{50}), and the K_l'-values corresponding to each inhibitor concentration are also shown in Fig. 2A.

When inhibitor concentrations are increased at constant [L] = up 40, there is strongly increased inhibition with a decrease in K_i (Fig. 2B). The inhibitor concentration [I_{50}] needed to reduce actual velocity to $V/2$ is shown in Fig. 2B for each value of K_i examined. Strong inhibition is produced in the marked mole fraction range of 1–4 mol%. This is the typical activity range of general anesthetics [1,2].

The influence of the number of lipid binding sites (n) was next examined (Fig. 2C). L_{50} is found to increase with increasing n, whereas I_{50} decreases with increasing n. The important result is that the ratio L_{50}/I_{50} which reflects inhibitor sensitivity strongly in-



creases with increasing n. A high number of 'boundary' lipid solvation sites is thus predicted to make functional membrane proteins much more sensitive to inhibition.

Discussion

The present results establish lipid/protein mismatch and displacement of 'boundary' lipid as a third candidate 'unitary' mechanism for non-specific xenobiotic inhibitors. Previous 'unitary' mechanisms were based on induced changes of membrane bilayer structure [1–3] and on substrate displacement [5]. All three mechanisms are much easier to demonstrate conceptually then to differentiate experimentally. The best experimental method to explore lipid/protein mismatch probably is EPR spectroscopy, since its time-scale allows to determine the number and relative affinity of 'boundary' lipid molecules [12–14,19]. An important functional restriction in the EPR studies on general anesthetics consisted in the need to record the spectra at non-physiological temperatures (0–7°C) [13,14].

In many functional membrane studies, added xenobiotics have initially stimulated the reaction and have

Fig. 2. (A) Lipid activation curves for a hypothetical 'average' membrane enzyme with $n = 20$ 'boundary' lipid-binding sites. Only the fully substituted enzyme species is considered to be active. Eqn. 2 is used with a value of $K_l = 0.1$. Lipid concentration is expressed as number of lipid molecules per protein molecule. In the absence of inhibitor, the parent curve shown at the top is obtained. The lower curves are calculated for increasing constant inhibitor concentrations of 0.5, 1, 4 and 12 inhibitor molecules per protein molecule. On the right hand side, the [I] values used, the L_{50} values and the apparent K_l-values, (K_l'), are shown for each curve. The parameters of the kinetic point marked by a full circle at 40 lipid molecules per protein molecule are used for the calculations of Fig. 2B and C. (B) Inhibition of a lipid-activated enzyme. The activated enzyme species defined by the full circle in Fig. 2A is examined in the presence of increasing inhibitor concentrations. These are indicated on the abscissa in units of inhibitor molecules per protein molecule, as well as in approximate mol fraction units relative to the lipid. Inhibition is examined for inhibitor binding constants of $K_i = 0.03$, 0.1, 0.3 and 1, the K_l value remaining constant at 0.1. The resulting inhibition curves are shown along with the K_i value used for calculation. On the right hand side, the I_{50}-values are indicated for each of the curves. The mol fraction region of 1–4 mol% is marked by a bar. This region is known to be critical for general anesthesia. (C) Inhibitor sensitivity of lipid-dependent enzymes as a function of the number of lipid activator binding sites (n). The activation point marked by a full circle in Fig. 2A serves as a reference for the calculations performed (that is, $L = 40$; $K_i = K_l = 0.1$; n being variable). L_{50} values at $I = 0$ were calculated from a relationship derived from Eqn. 2: $L_{50} = 0.1/(\sqrt[n]{2} - 1)$. For example, L_{50} values are 2.83 at $n = 20$ and 14.4 at $n = 100$. The I_{50} values were calculated from the following relationship which was again derived from Eqn. 2: $I_{50} = 40\ (\sqrt[n]{2} - 1.0025)$. I_{50} values were 1.3 at $n = 20$ and 0.18 at $n = 100$. Finally, the ratio L_{50}/I_{50} was plotted as a measure of inhibitor sensitivity. L_{50}/I_{50} had a value of 2.18 at $n = 20$ and of 80 at $n = 100$.

only inhibited at higher concentrations. This could indicate that initially the low degree of mismatch existing in normal membranes is relieved. In special cases, xenobiotics can be stimulatory over an extended concentration range. The stimulatory action of diethylether on vesicular Ca^{2+}-ATPase has been explained by a selective mobilization of lipid chains adjacent to the enzyme [20]. Amphiphilic xenobiotics, such as certain detergents, may even completely replace 'boundary' lipid activator molecules [7,9]. Organic solvents and chaotropic agents affected the lactose permease system of intact *E. coli* cells much more strongly than bilayer permeability. The induction of mismatch by increased lipid/protein dissociation constants was a likely explanation [21].

The stoichiometry between lipid activators and xenobiotic inhibitors is unknown at present and may greatly vary between lipoprotein systems. It is conceivable that a single xenobiotic molecule could displace more than one 'boundary' lipid molecule. Interestingly, the activity range of general anesthetics of 1–4 mol% in the membrane [1,2] is distinctly lower than the overall two-dimensional binding dissociation constant of about 20 mol% for the specific lipid activator of β-hydroxybutyrate dehydrogenase [17]. In view of the present lack of information, a 1:1 stoichiometry of lipid and inhibitor has been arbitrarily assumed in Fig. 2.

The strong influence of n on inhibitor sensitivity (Fig. 2C) is intuitively clear. L_{50} will strongly increase with n due to the increasing difficulty to fill all binding sites simultaneously. In contrast, I_{50} will strongly decrease with increasing n since inhibitor binding at a single site becomes much more likely. This statistical contrast between activation and inhibition leads to a high effectivity of the lipid/protein mismatch mechanism.

Induction of lipid/protein mismatch and subsequent inhibition of membrane functions could also be given by endogenous lipids such as sphingolipids, cholesterol or lysolipids. The highly irregular surface structure of proteins may always present problems for the packing and line-up of polar and non-polar groups of lipid and protein [7–11], but the lipid heterogeneity of biological membranes has been suggested to minimize lipid-protein mismatch [7].

A membrane enzyme may not strictly require full lipid substitution, but tolerate a certain number of vacancies [15]. In addition, lipid activation sites may show cooperative interactions [17,22]. Kinetic equations for such extensions of the present simple model have been developed [15,19,21,22]. The modelling power of the kinetic theory is illustrated by recent results. Theoretical analysis had predicted that the published extremely high cooperativity of the lipid activation of protein kinase C by phosphatidylserine [23,24] merely reflected an artifactual trapping process [25]. Recently, a Mg^{2+}-induced aggregation artifact has been reported to occur in the assay system. Aggregation had the same extremely high Hill coefficient for phosphatidylserine as observed for activation [26].

In conclusion, the present kinetic formalism appears to work for inhibitor membrane concentrations of 1–4 mol%, so that EPR-spectroscopic exchange parameters may now be linked mathematically to function.

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