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Kinetics of Binding of Membrane-Active Drugs to Receptor Sites

Diffusion-Limited Rates for a Membrane Bilayer Approach of 1,4-Dihydropyridine Calcium Channel Antagonists to Their Active Site

DAVID G. RHODES, J. GEORGE SARMIENTO, AND LEO G. HERBETTE

Departments of Medicine (D.G.R., J.G.S., L.G.H.) and Biochemistry (L.G.H.), University of Connecticut Health Center, Farmington, Connecticut 06032, and Department of Biology (D.G.R., L.G.H.), Brookhaven National Laboratory, Upton, New York 11973

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SUMMARY

Using the model of 1,4-dihydropyridine calcium channel-blocking drug binding to receptors in the cardiac sarcolemmal membrane, diffusion-limited rates of association were calculated for two distinct approaches. In the "aqueous approach," the drug reaches the receptor by diffusion through the bulk solvent, whereas in the "membrane approach," the drug partitions into the membrane bilayer and then diffuses laterally to a specific receptor site. Calculated rates for the membrane approach were approximately 3 orders of magnitude greater than those for the aqueous approach. The membrane approach diffusion-limited rate depends weakly on the sizes of the binding site, the drug molecule, and the vesicle, but depends strongly on ligand asymmetry. Although the measured binding rates for several 1,4-dihydropyridines were all slower than the calculated diffusion-limited rates for either model, other experimental data (such as very high partition coefficients and specific positions of these drugs in the membrane bilayer) suggest that the membrane approach is the most likely. These results have important implications for specifying critical characteristics of active 1,4-dihydropyridines.

INTRODUCTION

Several investigators have described the binding of DHP[1] calcium channel antagonists to specific receptors in a variety of tissues. Nitrendipine, one of these cardiovascular drugs, and other drugs of this class have been observed to bind with very high affinity (0.1–5 nM) to specific sites in membranes derived from cardiac, skeletal, and smooth muscle, and other tissues (1–4). To investigate the nature of the binding site, a wide variety of approaches has been used with varying degrees of success. Based on radiation inactivation experiments, it has been estimated that the molecular weight of the binding site molecule is 210,000–260,000 (5). The nitrendipine-binding site from rabbit skeletal muscle transverse tubule was recently shown to consist of three polypeptides of molecular weight 130,000, 50,000, and 33,000 (6). It has not been determined whether the 1,4-dihydropyridine-binding site is the calcium channel itself or an associated subunit of a more complex structure. Kinetic studies based on measurements of the time course of binding of radiolabeled analogs have been used to attempt to infer a general kinetic process by which DHPs bind to their receptor (1, 7, 8). The rapidity of these binding reactions and some additional observations from this laboratory have led to a consideration of the kinetics of the binding process from the perspective of diffusion-limited reactions, in order to obtain information related to a kinetic model for this binding process.

Relationships for calculating kinetic constants for diffusion-limited reactions have been reported for many different models (9–13). Whereas some models are attempts to correlate calculated and measured rates by restricting the reactants (14, 15), others consider complex approaches of reactants to each other, which result in dramatically increased predicted reactions rates (12, 13). Adam and Delbruck (9), for example, demonstrated that the predicted maximum rate was increased if the ligand was restricted to a two-dimensional surface containing the target site, thus reducing the translational states available to the reactants. Berg and Purcell (10) showed that, if one accounts for the fact that a ligand which collides with a surface (but not an active site on

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[1] The abbreviations used are: DHP, 1,4-dihydropyridine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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that surface) is likely to collide with that surface more than once before drifting away to some arbitrary distance, the calculated diffusion-limited rate is increased.

Several characteristics of the canine cardiac sarcolemmal membrane and the DHPs (to be discussed below) suggest that it is appropriate to consider an indirect, or two-stage, approach to the receptor site for binding of this class of drugs. In this "membrane approach," the drug approaches a vesicle, partitions into the lipid bilayer, and diffuses laterally to a receptor-binding site. Recent reports of patch clamp experiments with DHP derivatives using cultured rat myocardial cells (16) and neonatal rat or adult guinea pig ventricular cells (17) show that drug added to medium outside the patch affects single calcium channels within the patch (16). Since the receptor is physically isolated from the bulk solvent, the drug cannot use the aqueous approach. These observations strongly support the idea that DHPs reach their receptors on the calcium channel through the lipid bilayer.

In this report, calculations of the diffusion-limited rate for two different approaches of a drug to its binding site are presented and it is shown for the geometric model which best describes the *in vitro* DHP binding experiments, that 1) the membrane approach diffusion-limited rates greatly exceed all experimentally determined rates and 2) the direct (aqueous) approach to the binding site predicts much smaller maximum rates, although these are also greater than those measured experimentally.

MATERIALS AND METHODS

Sarcolemmal membrane preparation. Sarcolemmal membrane vesicles were prepared essentially according to the method of Jones *et al.* (18). Vesicles were formed by two 30-sec homogenizations with a Brinkmann Polytron at half-maximal setting. The homogenates were centrifuged at 21,000 × *g* for 30 min, the pellets were discarded, and the supernatants were recentrifuged at 45,000 × *g* for 30 min. These pellets, designated crude sarcolemmal vesicles, were resuspended in 0.25 M sucrose in 20 mM HEPES, pH 7.8 (buffer A). Crude sarcolemmal vesicles were frozen in liquid nitrogen and stored at −80°. All characterization assays were performed within 2 weeks of preparation, during which time no discernable deterioration of the samples (as assessed by several marker enzyme assays) was observed. Preparations typically contained 0.3 pM DHP receptor sites per mg of total protein and a total protein concentration of approximately 6 mg/ml.

Binding experiments. DHP-binding rates were measured by filtration assays similar to those used by Sarmiento *et al.* (7). Radiolabeled drugs ([^{3}H]nimodipine and ^{125}I-BAY P-8857: 2-iodoethyl isopropyl 1,4-dihydropyridine-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate) were incubated at 25° with 100-μg samples of sarcolemmal membranes in 150 mM NaCl, 10 mM Tris-Cl, pH 7.3 (buffer B) in 5-ml reaction volumes. With the resulting receptor site concentration of 6 pM, data were collected for drug concentrations ranging from 10 pM to 1 nM and for reaction times as short as 20 sec and as long as 150 min. Nonspecific binding was determined using 0.1 μM unlabeled nimodipine (7) in the reaction mixture. Samples were collected by filtering the reaction mixture through GF-B filters on a Brandell Cell Harvester and washing twice with 2.5 ml of buffer B. Specific binding was calculated by difference using the total binding data points and a best linear fit of the nonspecific data. Values for the forward rate, k_1, were calculated using a trivial modification of the analysis procedure of Weiland and Molinoff (8). Their Eq. 5 was rearranged to the form:

$$\frac{[LR]_E}{[L][R]} \ln\left(\frac{[LR]_E}{[LR]_E - [LR]}\right) = k_1 t \qquad (1)$$

where $[L]$ and $[R]$ are the total concentrations of drug and receptor, respectively, $[LR]_E$ is the equilibrium concentration of ligand-receptor complex, and $[LR]$ is the concentration of ligand-receptor complex at time t. The forward rate was determined directly as the slope of a plot of the left side of Eq. 1 as a function of time.

Partition coefficients. Partition coefficients were measured by a centrifugation assay developed in this laboratory to measure partition of a substance into a lipid phase, rather than simply into an organic phase.[2] Various concentrations (0.01 nM to 1 μM) of isotopically labeled DHP were added to polyethylene Microfuge tubes containing suspensions of sarcolemmal membranes (19). After the samples had equilibrated for approximately 24 hr at 5°, they were centrifuged at 25,000 rpm for 1 hr. The pellet region of the tube was cut off with a razor blade and blotted dry, aliquots of the supernatant were collected, and the remainder of the tube was counted to assess radioactivity bound to the tube. Because the water volume in the pellet is very small (20) and the aqueous solubility of these drugs is very low, it was not necessary to correct for labeled drug trapped in the interstitial water spaces in the pellet. Blank tubes (without membranes) were treated in a similar manner in order to correct for adhesion of the drug to the walls of the tube in the pellet region. The partition coefficients [g(drug)/g(lipid)/g(drug)/g(aqueous solvent)] were determined by measurements of radioactivity associated with the pellets and the supernatants. Over this range of drug concentrations (0.01 nM to 1 μM), the partition coefficient was found to be constant, as one would expect for a simple solute. This partition coefficient is likely to be the true lipid partition coefficient since 1) the lipid-to-protein ratio is relatively high, so that the major contribution to nonspecific binding is from association with lipid, and 2) the pellet is not washed, so that the nonspecific binding is far greater than the specific binding over the entire concentration range.

SPECIFICATION OF THE SYSTEM

The DHP/Sarcolemmal Receptor System

Equilibrium and kinetic binding characteristics of the DHP/sarcolemmal membrane receptor system have been reported (1, 2, 7). In order to define clearly the chemical system to be modeled, it is appropriate first to review certain pertinent information regarding the physical and chemical properties of this system.

Kinetics. Most kinetic studies of drug interaction with membrane-associated receptors rely either on measurement of the time course of specific binding of radiolabeled drug analogs (1) or on measurement of the kinetics of specific ion fluxes in electrophysiological experiments (21). According to the measurements of Bolger *et al.* (1), the overall forward rate constant, k_1, for [^{3}H]nitrendipine binding to specific receptors isolated from guinea pig ileal smooth muscle is approximately 9×10^6 $(M \cdot sec)^{-1}$. With canine cardiac sarcolemmal receptors, it has been determined that k_1 for nimodipine is 1.0×10^7 $(M \cdot sec)^{-1}$ and k_1 for BAY P-8857 is 2.7×10^7 $(M \cdot sec)^{-1}$ (22). These rate constants are consistent with those obtained in this laboratory for other DHPs, but because of technical limitations to the radiolabeled ligand-binding assay, it would be difficult to measure rates faster than these using this method, so these should be considered minimum values.

[2] This procedure will be described in greater detail in a later report. Measurement of the partition coefficients reported here depends on the fact that the lipid component (rather than the protein component) dominates the total nonspecific binding. Although the protein nonspecific binding depends strongly on the particular drug, the total nonspecific binding may be considered as due entirely to the lipid component.

Physical Properties

The receptor. Work by Colvin *et al.* (19) has shown that the lipid/protein ratio in the canine cardiac sarcolemma is very high, approximately 3000 nmol of lipid/mg of protein. This ratio suggests that a typical area of membrane would contain large areas of lipid with proteins inserted at widely separated intervals. Most of the protein present is Na/K-ATPase, and relatively little represents specific DHP receptor. The concentration of ouabain-binding sites (hence Na/K-ATPase), for example, is 400 times that of DHP-binding sites in this membrane system (19). Taking the area of a typical lipid molecule to be 60 $Å^2$ and knowing the lipid-to-protein ratio (3000 nmol/mg) and maximum equilibrium binding values for these calcium channel antagonists (1 pmol/mg of protein), one may calculate that the number of receptor sites per unit area is less than 1 site/μm^2 (19). This information, taken with the molecular weight and a reasonable assumption for the density of the protein complex associated with this site, indicates that only $1/_{35,000}$ of the total membrane area is receptor. From the surface density of sites, a vesicle of radius 2821 Å would be expected to contain exactly one site. Since this is a reasonable size for vesicles of this type, as a simplifying assumption for many of the model calculations presented below, vesicles are taken to be uniform in size and of radius 2821 Å (see Discussion).

The ligand. Of the numerous derivatives of the DHPs, only a few have received critical analytical attention (23, 24). Generally, the molecular weight is in the range 400–500, and for hydrodynamic considerations, the basic two-ring structure is represented fairly well as an oblate ellipsoid with major and minor semi-axes of 6 and 4 Å, respectively. Small alkyl substitutions at the 3- and 5-positions (e.g., nitrendipine, nimodipine, nifedipine, nisoldipine) do not significantly alter this shape, but with larger substitutions (e.g., nicardipine), the structure is better represented as an asymmetric prolate ellipsoid. For the model calculations presented here, the ligand is assumed to be spherical, an approximation which introduces an error of less than 3% to calculations of the limiting rates for the aqueous approach. Since only limited information is available regarding the orientation of the ligand in the membrane, it is impossible to estimate the error in lateral diffusion rates using this simplified geometry.

It is well known that DHPs are readily soluble in organic phases (23, 24). For several DHPs, nimodipine, iodipine, and BAY P-8857, partition coefficients [g(drug)/g(lipid) ÷ g(drug)/g(aqueous solvent)] into the sarcolemmal membrane have been reported to be 4,100, 17,500, and 100,000, respectively (22). These are relatively high partition coefficients compared to those that have been measured for other drugs which act on membrane-bound receptors. Propranolol, a membrane-active drug whose partitioning has been analyzed in terms of a detailed chemical and structural analysis, has a partition coefficient of approximately 200 and timolol, another β-blocker, has a partition coefficient of approximately 20 (20).

Although the DHPs do partition into the membrane bilayer, it is not known whether these ligands orient selectively, as has been observed for other membrane-active drugs (20, 25). Certainly, the orientation of these ligands, their position in the bilayer, and the homogeneity of this orientation will depend on the substitutions present at the 3- or 5-position of the pyridine ring. This fact may well be related to the observation that for very subtle changes in the 3- or 5-substitutions of various DHPs profound differences in rates of binding and activity (IC_{50}) are observed (24). For some other drugs which act on membrane-associated receptors, the site of nonspecific association has been identified as a well defined position in the membrane, relative to the bilayer normal (20). (The bilayer normal is defined as an axis perpendicular to the plane of the phospholipid head groups and parallel to the fatty acyl chains.) Although many such ligands will not extend from the membrane surface to the center of the hydrocarbon core, the disorder imposed upon the monolayer occupied by the ligand is likely, on average, to display cylindrical symmetry about the bilayer normal. Therefore, for the purposes of this derivation, the two-dimensional translational and rotational diffusion relations for ideal cylinders (28) have been used. It is recognized that the lateral diffusion may not display the behavior predicted by the Stokes law model, and that particular intermolecular interactions may reduce the rate of diffusion, but the present treatment is for ideal diffusion-limited rates, and neglects such complications.

The derivation, which appears in the Appendix, is based on a simple model, shown in Fig. 1. To simplify the derivation, only two of the possible approaches shown in Fig. 1B are considered. In the aqueous approach, a ligand reaches its receptor directly (with a mean time to collision of τ_3), whereas in the membrane approach the ligand first partitions into the membrane bilayer (with a mean time to collision of τ_3') and then diffuses laterally to the receptor (with a mean time to collision of τ_2). The net collision time becomes (as seen in the Appendix)

$$\tau_{32} = \frac{c^3}{3bD_3} + \frac{b^2}{D_2}\left(\log\frac{4b^2}{a^2} - 1\right) \qquad \text{(A5)}$$

where a is the receptor radius, b is the vesicle radius, c is the radius of a spherical solution element containing one receptor, D_3 is the spatial diffusion coefficient of the ligand, and D_2 is its lateral diffusion coefficient. (See Appendix for further details.) The significance of this result and the sensitivity of the dependence of τ_{32} on various molecular parameters is considered in Results.

RESULTS

Model calculations. To better understand the implications and limits of this model and to assess the severity of the assumptions made in assigning numerical values to molecular parameters, several of these parameters were varied systematically. Since some of the parameters which describe the system affect more than one part of the overall reaction, it is not immediately apparent which parameters most strongly affect k_{32} and, therefore, for which parameters accurate quantitation is most critical. None of these calculations attempts to account for partially reactive sites [e.g., by using the κ factor invoked in

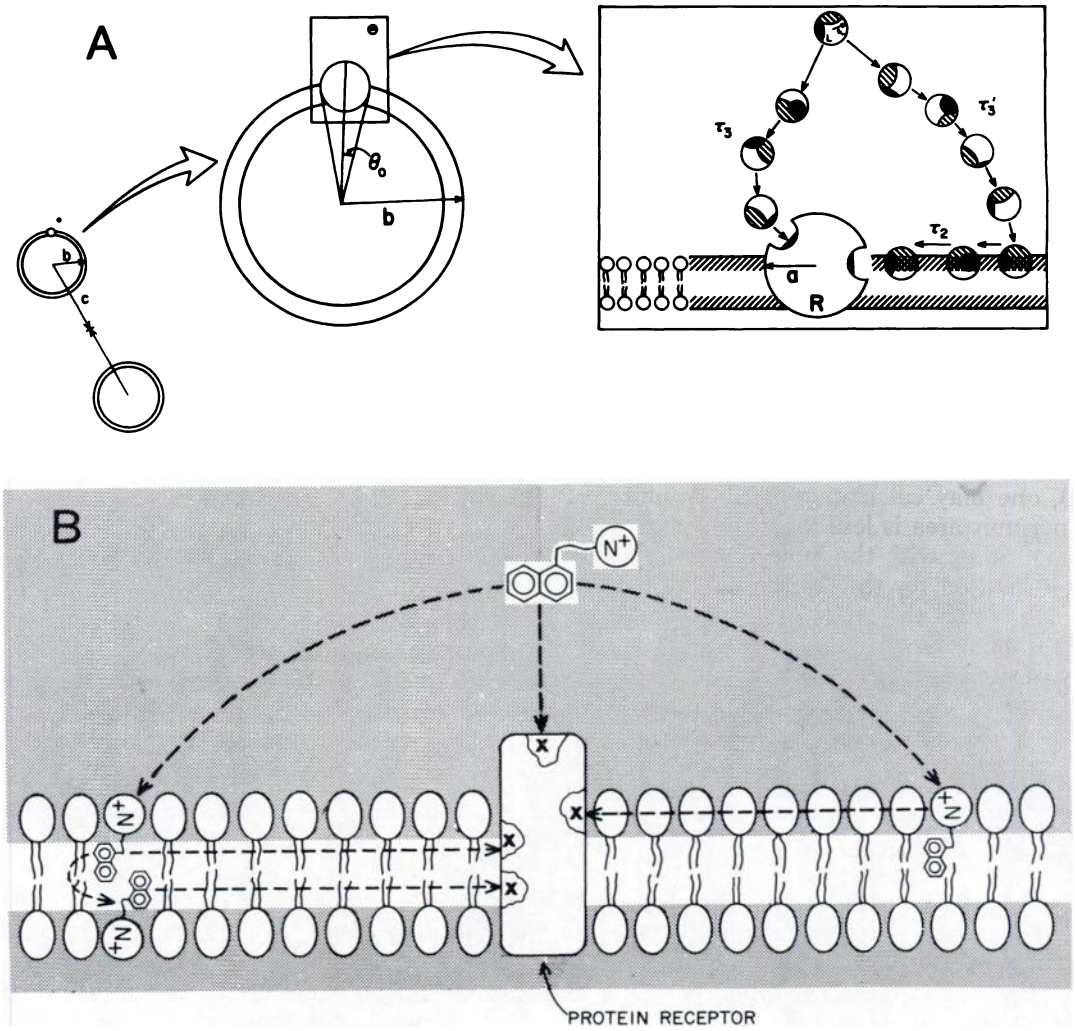

FIG. 1. *Schematic diagrams of a ligand binding to a receptor*

A illustrates several of the parameters used to describe the model. Vesicles in solution are each allocated a spherical volume element of radius c. The vesicle radius is b. A receptor (R) of radius a may have a binding site accessible either to the solvent or to the bilayer. A ligand molecule (L) of radius r_L reaches a binding site on the portion of the receptor in the aqueous environment with a mean time to collision of τ_3. The alternative approach involves partitioning, with a mean time to collision of τ_3', and lateral diffusion to a binding site on the portion of the receptor in contact with the bilayer, with a mean time to collision τ_2. Here, θ_0 corresponds to the Solc and Stockmayer (15) asymmetry parameter, which was also used for the Weaver-type (30) calculation of τ_2. B is a generalized diagram in which several potential approaches for a ligand to bind to a target receptor site are illustrated. The direct approach through the aqueous solvent (gray area) is to a site on the hydrophilic portion of the receptor. The membrane approach may be to a site on either a hydrophilic (right side) or a hydrophobic portion (left side) of the receptor. As shown on the left side, a ligand could diffuse to the inner monolayer, assume an energetically favorable position in either the hydrophobic (shown) or hydrophilic (not shown) region of the inner monolayer, and diffuse laterally to the receptor site. For simplicity, the latter approach (flip-flop) was not considered in the derivation.

other derivations (29, 30)], or for kinetics of microscopic reaction steps involving energetic, rather than diffusive, molecular processes (e.g., ligand partitioning or changes of receptor structure). Although the predicted rates which result may be closer to measured values, these are not true diffusion-limited rates. In addition, there has been no consideration of chemical nonideality, aspherical reactants, or microscopic heterogeneity of membrane structures (31).

Since the relationships are intended to apply to a wide range of related dihydropyridine derivatives of various sizes, one of the first parameters to consider is ligand radius. Fig. 2 shows the dependence of the diffusion-limited rate as a function of ligand size for several degrees of asymmetry for both the aqueous and membrane approaches. Calculations for the membrane approach require specification of the vesicle size and concentration, so, for simplicity, the vesicle radius was used which corresponds to one receptor site per vesicle (b = 2821 Å) at a site concentration of 2 pM. Several interesting features are evident.

1) The rate decreases with increasing ligand size in all cases. This is a reflection of the fact that the contribution to k by the translational diffusion coefficient is dominated by the ligand diffusion coefficient, which is inversely proportional to the ligand size, whereas the radius term in k is dominated by the larger target radius, and is nearly independent of the ligand size.

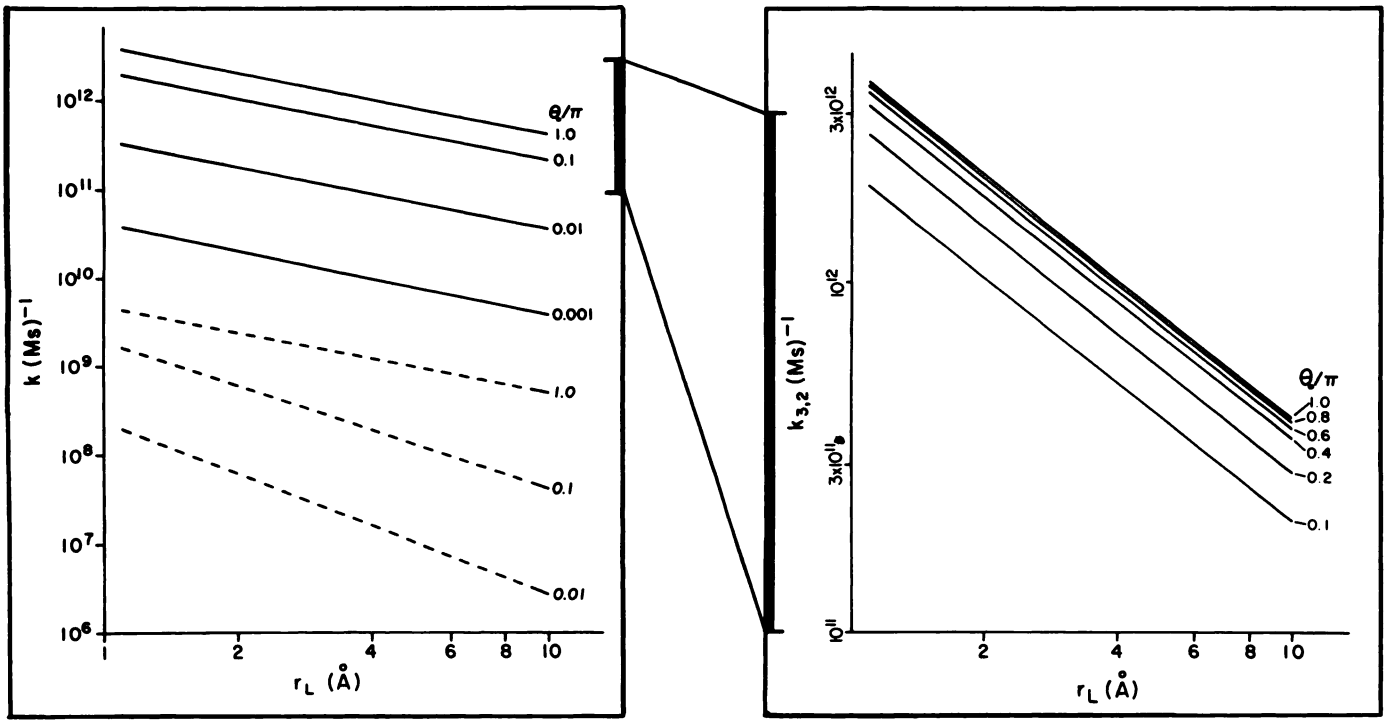

FIG. 2. *Dependence of the diffusion-limited forward rate on ligand size*

Solid lines are calculations for the membrane approach; dashed lines are for the aqueous approach. These calculations are for $\theta_0 = \pi$ (isotropic) to $\theta_0 = 0.001\pi$ (very restrictive orientation constraint) and for $\theta_0' = \pi$ to $\theta_0' = 0.01\pi$. All curves are for a = 6 Å, b = 2821 Å. Clearly, for comparable parameter sets, $k_{32} \gg k_3$.

2) $k_{32} \gg k_3$. The limiting rate for the membrane approach is approximately 3 orders of magnitude faster in all cases, and for no set of parameters was $k_{32} < k_3$. As an indication of the magnitude of the difference between k_{32} and k_3, note that one must approach the limit of absolute orientational constraint for k_{32} to approach the isotropic limit for k_3.

It is interesting to note the mean times to collision for purposes of comparison. Taking a ligand of radius 6 Å at the isotropic limit ($\theta = \pi$), the mean time to collision (τ_{32} = 0.74 sec) is dominated by the approach to the vesicle (τ_3' = 0.66 sec). For a highly anisotropic ligand ($\theta = 0.01\pi$) under the same conditions, however, τ_2 becomes very large (7.96 sec) compared to τ_3' (0.66 sec).

The calculations in Fig. 2 are for a fixed vesicle size, b = 2821 Å. By the nature of the model, k_3 does not depend on the size of the vesicles, but there is a marked dependence of k_{32} on b. With larger b, k_{32} would be expected to increase since the size of the target for the first phase (partitioning) is increased, but k_{32} would decrease due to increased τ_2 caused by an increased area in which the receptor could be found. Fig. 3 illustrates the effect of these antagonistic factors. For vesicles of reasonable size, the effects of vesicle size on τ_2 are apparent only for ligands with a relatively high degree of anisotropy. For a greater degree of anisotropy, the effect of b on τ_2 overcomes the effect on τ_3', evident at smaller b. In the small vesicle limit, τ_2 becomes much smaller than τ_3' so that k_{32} becomes insensitive to θ and all curves converge.

For most biological preparations, including cardiac sarcolemma, the vesicle population is heterogeneous, so the vesicle radius is an ill defined parameter. A broad distribution of vesicle sizes (radii from 250 to 3000 Å) is observed in thin section electron micrographs of canine cardiac sarcolemmal preparations (19). For such a heterogeneous population, the measured forward rate, assumimg the membrane approach to be operative, would be essentially a number average forward rate dependent on the distribution of vesicle sizes within the heterogeneous population. This consideration is insignificant for the present purpose, however, The dependence of k_{32} on b is relatively weak, varying as little as 2-fold over the observed range of b, so the choice of vesicle size in model calculations is probably not critical.

The insensitivity of k_{32} to θ at small b is apparent if one considers the dependence of k_{32} on θ directly. Fig. 4 illustrates the strong dependence of k_{32} on the degree of anisotropy for larger vesicles and the relatively weak dependence for small vesicles. The intersection of these curves reflects the fact that the curves in Fig. 3 are not monotonic. Although not illustrated here, there is a finite rate of reaction, even for $\theta = 0$ since a ligand may rotate during an encounter so as to achieve proper orientation, albeit for only an infinitesimal time.

These anisotropy parameters, θ and θ', are very difficult to evaluate experimentally, but have a strong influence on the forward rate. To consider only the isotropic extreme, $\theta = \pi$, however, will result in an overestimate of the diffusion limit since the ligand is obviously asymmetric and has clearly defined critical groups.

For the calculations presented above, the site size has been fixed at 6 Å. This approximation was based on the

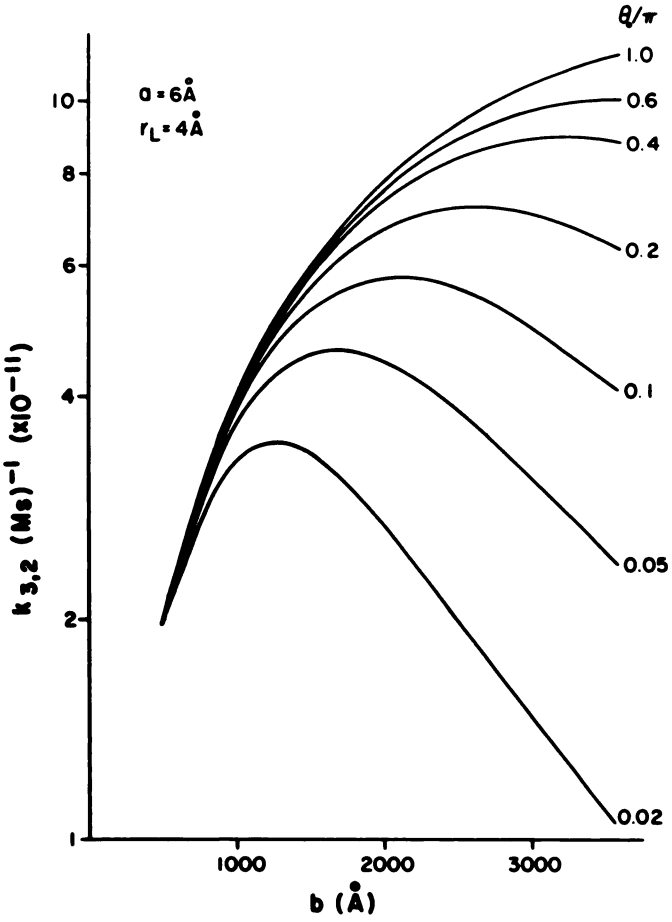

FIG. 3. *Dependence of the membrane approach diffusion-limited rate on the vesicle radius*

Increasing the size of the vesicle both increases τ_2, by increasing the total available surface area in which the receptor could be found, and decreases τ_3', by increasing the size of the target for this step. For small vesicles and for nearly isotropic ligands, τ_3' dominates, so that k_{32} increases with b. As the anisotropy increases and b becomes larger, however, τ_2 becomes significant and k_{32} decreases with b.

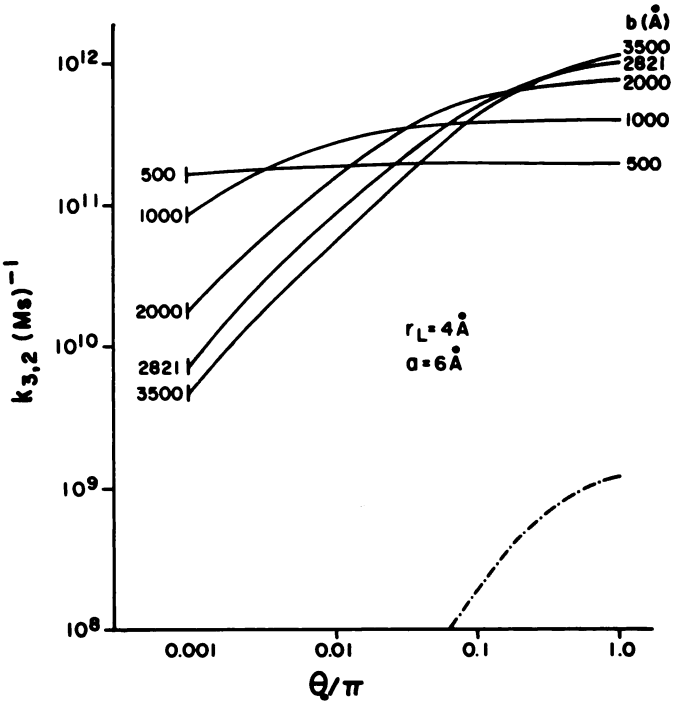

FIG. 4. *Dependence of the membrane approach diffusion-limited forward rate on ligand acceptance angle θ*

Curves for several vesicle radii were calculated at fixed site and ligand radii, for acceptance angles from $\theta = \pi$ to $\theta = 0.001\pi$ (very restrictive) and at $\theta = 0$. For highly anisotropic ligands, τ_2 becomes large so k_{32} varies inversely with b. As $\theta \rightarrow \pi$, τ_2 becomes small, so k_{32} increases with b. Also shown (–·–) is the θ' dependence for the aqueous approach to the site.

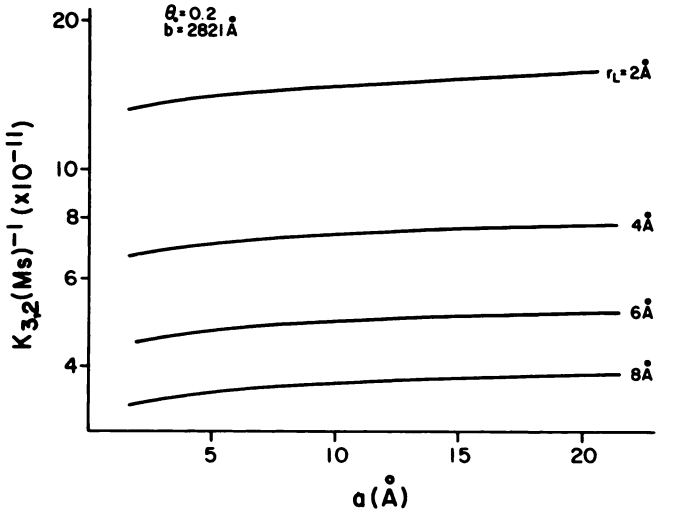

FIG. 5. *Dependence of the membrane approach diffusion-limited forward rate on the size of the binding site*

The dependence of k_{32} on a is very weak. (Compare, for example, to Figs. 2, 3, and 4.) Therefore, the choice of a = 6 Å used in many calculations here does not significantly affect the result.

estimated effective radius of DHPs using space-filling models and X-ray diffraction data (32, 33) and on the assumption that the target for the ligand, be it a cleft in the receptor protein surface or a set of favorably situated amino acid R-groups, should be approximately the same size as the ligand itself. Certainly, favorable charge interactions would increase the effective target radius, probably to something on the order of a Debye length, but DHPs generally have no formal charge, so it is unlikely that charge effects are directly involved in ligand-receptor interactions. This justification aside, the dependence of k_{32} on a is very weak (Fig. 5). For ligands of radius 4–6 Å, a 10-fold increase in site radius only increases k_{32} by approximately 15%. Since the dependence of k_3 on a is linear, a influences k_3 more strongly than k_{32} (see Discussion).

For all of the calculations above, the distribution of binding sites was considered to be perfectly uniform. Suppose that the sites were distributed such that some vesicles contained no sites while others contained multiple sites. The area density of sites is a known, measured parameter and does not vary. One would expect a faster predicted rate since the τ_2 on multiple site-containing vesicles is smaller, but a slower predicted rate since receptor collisions with non-site-containing vesicles are unproductive. For site distributions with nonuniformity as high as 10:1 (10 vesicles with no sites for every one

vesicle with 10 sites), calculated diffusion-limited rates did not vary more than 1% from those calculated for uniform site distribution in any case tested (data not shown). Clearly, in extreme cases, the functional form of the dependence of k_{32} on the various molecular parameters discussed above would not be the same as in the uniform site distribution case. As the distribution becomes highly nonuniform, τ_2 becomes negligible and the system begins to resemble isotropic, highly diffuse receptors with effective target radius nearly that of the vesicle itself. Such extremes are unlikely, however, and were not considered further.

Measured rates. Rates of specific DHP binding determined in the present study were consistent with reported rates, which are in the range 2×10^5–1×10^7 $(M \cdot sec)^{-1}$ (34). Rates for the two compounds tested, nimodipine and BAY P-8857, were found to be 1.0×10^7 and 2.7×10^7 $msec^{-1}$, respectively (Fig. 6). The rate for nimodipine is equal to that reported for binding of nitrendipine to receptors from rat brain (4), and the rate for BAY P-8857 is somewhat faster than this, the fastest reported rate thus far for a drug of this class.

These measured rates are slower than the isotropic diffusion-limited rates for either the aqueous approach or the membrane approach. Nevertheless, since the membrane approach diffusion-limited rates are approximately 1000 times faster than those for the aqueous approach, further consideration should be given to such details as the microscopic energetics of the binding reaction and likely limits to the reactivity of asymmetric ligands. A detailed comparison of the measured rates to the calculated diffusion-limited rates will be presented in Discussion.

DISCUSSION

Many members of the DHP class of calcium channel antagonists and agonists exhibit features which can be explained by, or are consistent with, the membrane approach described above. The concept of an approach to a site by some route other than directly through aqueous solvent is not, in itself, novel. Some descriptions of multiple step approaches to a binding site refer to excursions along a surface, with ligands making multiple unsuccessful collisions with inert portions of an anisotropic target before reaching the active site (10, 35). Others have described surface diffusion components in which the ligand is loosely associated with the inactive portion of the receptor surface by charge (12, 13, 36) or other (unspecified) interactions (9). In other reports, investigators have presented evidence for lateral movement of components constrained in membranes (37) and for the approach of lipid-soluble drugs to a binding site on a membrane-associated receptor through the nonpolar bilayer core (21).

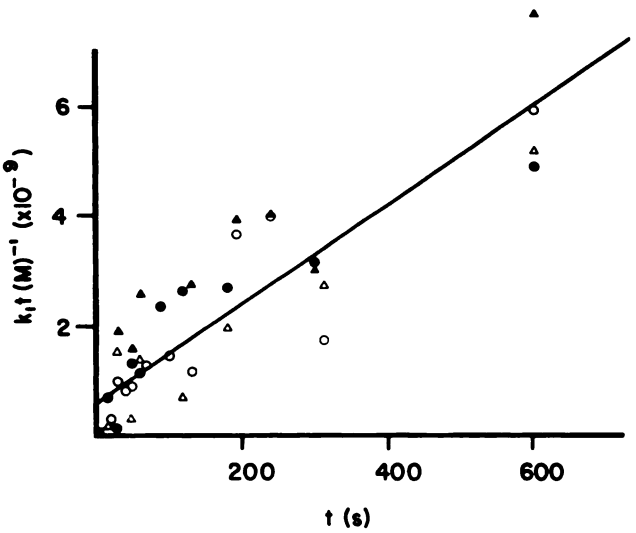

FIG. 6. *Kinetic data for [^{3}H]nimodipine plotted according to Eq. 1* Data are from four experiments at the following drug concentrations: ▲, 1×10^{-11} M; ●, 7×10^{-11} M; △, 1×10^{-10} M; ○, 7×10^{-10} M. The slope gives k_1 directly, in this case 9.2×10^6 $(M \cdot sec)^{-1}$. Data from approximately 20 such experiments on five different preparations resulted in the forward rate constant cited in the text.

Several features of the canine cardiac sarcolemmal membrane DHP receptor suggest that the membrane approach to the binding site is most likely. The DHPs are very lipid soluble and although they are chemically asymmetric, few could be classified as amphiphilic. Therefore, in addition to the existence of a high equilibrium partition coefficient, this equilibrium is likely to be established quickly. In addition, the receptor site density is very low, so that probability strongly favors collision with a non-receptor protein portion of the membrane. Since the membrane solubility of these drugs is so large, partitioning is likely, and it should not be surprising to find this step as part of a binding mechanism. Finally, these drugs are apparently located at a specific position in the membrane along the axis normal to the plane of the bilayer. One amphiphilic cardiac drug, propranolol, has been shown to position and orient specifically in a model membrane devoid of specific binding sites (20). Although orientation has not been demonstrated for any DHPs, X-ray diffraction and neutron diffraction studies on two different DHPs suggest that the pyridine ring is at approximately the same position in the membrane bilayer as the naphthalene rings of propranolol (22).

Is the reaction diffusion limited? If the assigned values for the various molecular parameters are reasonably close to the true values, these predicted maximum rates may be compared to the measured rates to assess the extent to which these binding reactions are diffusion limited, and to see whether it is reasonable to discount either approach on this basis. If the ligands nimodipine and BAY P-8857 are assumed to have effective radii of 5 Å, the measured rates for these compounds (1.0×10^7 and 2.7×10^7 $(M \cdot sec)^{-1}$, respectively) may be compared to the calculated curves in Fig. 2. Both rates are far below the isotropic diffusion-limited rate for either approach. If all of this difference is attributed to ligand anisotropy, θ_0' for the aqueous approach would be 0.01π for nimodipine and 0.03π for BAY P-8857, but even with $\theta_0 = 0$ for the membrane approach, the diffusion-limited rate exceeds the measured rates for both compounds. Clearly, the binding process is not diffusion limited. Since these diffusion-limited rates refer only to the binding process (rather than to a "result" of binding, such as opening of a channel), there must be one or more processes which

occur before the binding reaction, the rate(s) of which must be taken into consideration. There is at least one report (4) that an isomerization of the protein occurs before binding. This conclusion was based on comparison of observed binding data with binding curves predicted from several models, with/without isomerization(s) before and/or after binding. It is possible to interpret the membrane approach in terms of these experiments. The ligand partitioning into the membrane could be considered, in effect, an "isomerization." The concentration and temperature dependence of these two possible first steps, isomerization or partitioning, may eventually provide the distinction necessary to discern these two possible mechanisms.

Some effects have been neglected which could alter k_{32}. So that the true diffusion-limited rate is actually considered, imperfect reactivity at the site has been neglected. Such effects might be expected since there is some evidence that DHPs bind only to one of at least two protein conformational states (4). Similarly, partitioning into the membrane bilayer has been assumed to be instantaneous. Kinetic studies with model membranes should provide sufficient data to allow the kinetics of partitioning to be incorporated into this model, a refinement that would decrease k_{32}. Consideration of multiple collisions in the lateral diffusion step would increase the estimate of k_{32}. Fig. 7 illustrates that the effect of the latter refinement is most pronounced for highly anisotropic ligands. According to the theory of Buas (35) the mean number of collisions for the system parameters used here is approximately 7, the factor by which the two curves differ as they become parallel at small θ.

The ligand in the bilayer. Elaborations on the model as presented could also affect the measured rate. Fig. 1B shows a ligand/membrane/receptor system with four possible approaches to an active site. Thus far, only the aqueous approach and the membrane approach, in which the ligand partitions to the same level as the active site relative to the bilayer normal, have been considered. In the membrane approach, it is not important whether the active site on the receptor is in the polar or the nonpolar region of the bilayer, as long as the position of the receptor site along the bilayer normal corresponds to that of the partitioned ligand. If these positions do not align exactly, binding would depend on diffusion perpendicular to the plane of the bilayer, a thermal "bobbing" about the mean position. The effect would be to slow the forward rate, as fewer of the drug-receptor collisions would be successful.

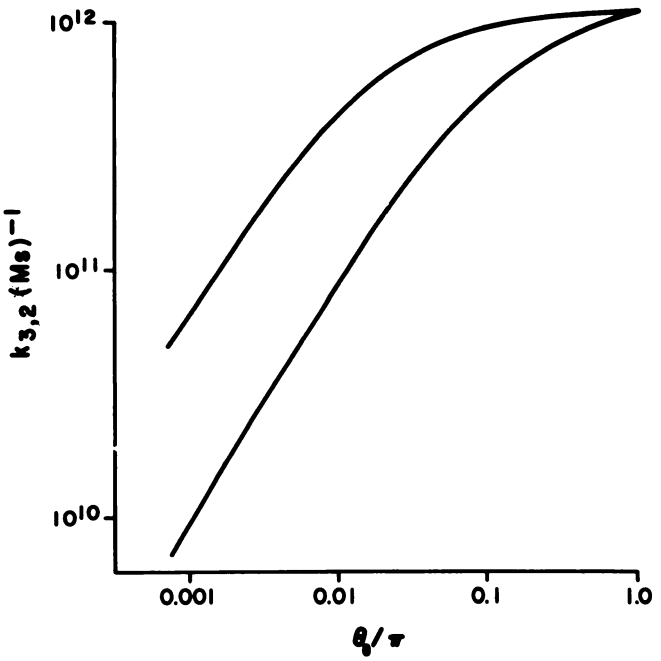

FIG. 7. *Enhancement of two-dimensional collision rate by multiple collision correction*

The lower curve is equivalent to the trace in Fig. 4 for b = 2821 Å. The upper curve is for the same set of parameters, but with a correction for multiple collisions in the membrane approach to the site (35).

It is implicit in the two-dimensional mathematics of the membrane approach model that the drug molecule is properly oriented for binding relative to the bilayer normal axis and that the conformations of the drug and its binding site are compatible. Once the ligand has partitioned into the bilayer, all rotational motion other than that about the bilayer normal axis is neglected, as are conformational changes. It is possible that the well defined structure of the lipid bilayer constrains active drugs not only to a well defined position along the bilayer normal, relative to the active site, but also to a favorable orientation and conformation. Again, if the equilibrium orientation and conformation are not "correct," binding would depend on random deviations from equilibrium and the measured rate would be lower than the diffusion-limited rate.

Another possibility not considered in the rate calculations is that of "flip-flop" of the ligand from one monolayer to the other. Some ligands could traverse a bilayer in this manner, with some relaxation time τ, to assume a position in the opposing monolayer. If the binding site on the receptor were at this position within the inner monolayer, the predicted rate would probably be decreased considerably to allow for this additional step.

Dissociation and in-membrane concentrations. Although the diffusion-limited rate does not specifically address the dissociation rate, the conclusion that the membrane approach is likely does carry certain implications regarding dissociation. If the approach to the site is through the bilayer and the equilibrium partition coefficient is high, it is reasonable to expect that an equilibrium exists between drug bound to receptor and drug in the membrane. (The drug in the membrane is, in turn, in equilibrium with the drug in aqueous solution.) In order to determine the true equilibrium binding constant or intrinsic forward rate constant for this reaction step, one must evaluate the ligand concentration in the membrane. The usual approach to this problem (38) has been to consider the ligand concentration relative to the total membrane volume. If the ligand is constrained at a particular level in the bilayer, this calculation would be an underestimate of the true concentration by a factor equal to the ratio of the membrane width to the mean distance of fluctuation of the ligand along the axis normal

to the bilayer plane. While this consideration could be interpreted as implying a weaker association than had been previously measured, it might also be said that the effective concentration of ligand at the level of the binding site is much higher (assuming the site to be at the same level).

The use of mean times to collision in the present derivation avoids this dilemma. As discussed above, assuming that the movement in the membrane is well represented by two-dimensional geometry asserts that the ligand and site are at exactly the same position along the bilayer normal and that the ligand is properly oriented relative to this axis. When accurate data are available regarding not only the positions of the ligand and its binding site, but also the kinetics and energetics of deviations from this mean position, the two-dimensional approximation may be discarded in favor of a more sophisticated model.

Implications for drug design. This binding model has profound implications relating to the design of new drug compounds. If the membrane approach is operative, the chemistry of the partitioning process should be an important determinant of drug activity. Different derivatives in a class of drugs, when partitioned into a bilayer, may be at different positions along the bilayer normal, may have different orientations, may have different basic structures, and may have different equilibrium membrane concentrations. For example, a DHP with an extended aliphatic substitution at the 3- or 5-position, which is positioned at the level of the active site in the bilayer, may exhibit long term residual activity whereas a less lipid-soluble ligand may dissociate more quickly. Differences in the apparent binding constants could actually reflect differences in the level at which the ligands partition in the membrane, thus altering the effective concentration available to the active site. Likewise, these fundamental effects could produce very different IC_{50} values.

Conclusion. Although available data do not show conclusively that the membrane approach is operative in the case of DHP binding to sarcolemmal receptors, the discussion above and the work of others (16, 17) suggest strongly that this approach is likely. Current research in this laboratory, using spectroscopic analysis, will allow the binding reaction to be followed on a much faster time scale and should provide detailed information on the chemical environment of the DHP molecules as they approach the receptor site and bind. This information will confirm or refute the assertion that DHP compounds approach receptors through the membrane bilayer. If the membrane approach proves to be the operative mechanism for this or other drug/receptor reactions, it may become important to consider not only the conformation of a drug in an aqueous or crystalline environment, but also the (perhaps different) conformation of the drug dissolved in a membrane bilayer. By studying the structure of drugs in their "natural" environment, it may be possible to design new drugs with greater specificity and effectiveness.

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APPENDIX: DERIVATION OF MODELS

The choice of a simple but accurate model is a crucial aspect of any theoretical calculation. In the present case, the basic system consists of (Fig. 1) receptor sites of radius a, situated on spherical vesicles of radius b, which are c apart in solution. (A list of variables is found in the Glossary following). A ligand, of radius r_L, reaches the target site by either the aqueous approach or the membrane approach with a rate for each characterized by a particular mean time to collision, τ. Equations for calculating diffusion-limited rates are derived below for each approach to the receptor.

The aqueous approach. For the present treatment, the Schmitz and Schurr (14) model of an asymmetric ligand reacting with an isolated circular active site on an otherwise inert planar surface has been used (see Fig. A1). Since the surface area of sarcolemmal vesicles is so large relative to that of the receptor protein-binding site, and since the site density is so low (1 site/μm^2), this receptor/

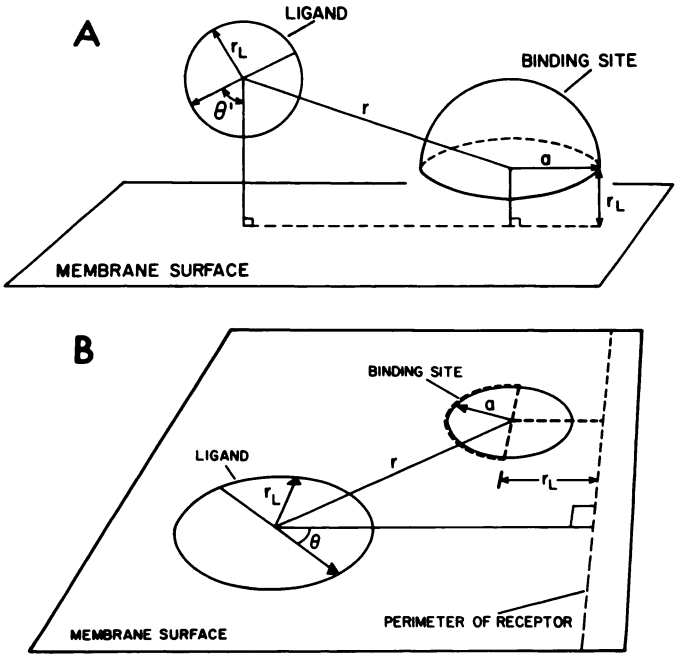

FIG. A1. *Ligand-receptor geometry for three-dimensional (A) and two-dimensional (B) diffusion*

Both models are based on the model of Schmitz and Schurr (14). The asymmetric ligand of radius r_L approaches the target site of radius a. In three dimensions (A), the ligand orientation is described by an angle θ' relative to a normal to the bilayer plane containing the receptor site. B illustrates the manner in which this geometry was adapted for application to the two-dimensional case. In two dimensions, the active site becomes a semicircle about an active site (of length $2a$) on an infinite line, which could represent a portion of the periphery of a large protein complex. The orientation of the ligand is described by an angle θ relative to the normal to this line.

ligand system is approximated quite well by the model of an isolated patch on a plane. Eq. A1 is the Shoup *et al.* (29) expression of the diffusion-limited rate for the Schmitz and Schurr model in the limit of complete diffusion control.

$$k_3 = \frac{4\pi D_3 a(1 - \cos\theta_0')^2}{\sum_{l=0}^{\infty} \frac{[p_{l+1}(\cos\theta_0') - p_{l-1}(\cos\theta_0')]^2}{(l + 0.5)(l + \xi)}} \quad \text{(A1)}$$

Here, a is the radius of the site, D_3 is the ligand diffusion coefficient, P_l is the lth Legendre polynomial, θ_0' is the acceptance angle for the anisotropic ligand, $\xi = b[l(l + 1)D_R/D_3]^{1/2}$, b is the vesicle radius, and D_R is the rotational diffusion coefficient. For reaction to occur, the angle between the ligand orientation vector and a normal to the plane containing the site (θ') must be less than or equal to an acceptable angle, θ_0'. In addition, the ligand center must lie within the hemisphere of radius a positioned r_L above the plane. For purposes of comparison, Eq. A1 has been used to calculate diffusion-limited rates for the aqueous approach for the same parameter sets used to calculate limiting rates for the membrane approach.

The two-stage membrane approach. In the membrane approach, the ligand first approaches the vesicle in bulk solution and, upon collision with the vesicle, is incorporated into the membrane bilayer. The ligand then moves laterally through the membrane bilayer until it collides with a receptor embedded in the bilayer. For the present discussion, it is assumed that all drug-membrane collisions are successful and immediate. Therefore, to estimate a diffusion-limited rate, it is only necessary to consider two processes, that of diffusion to the vesicle through the bulk solvent and that of lateral diffusion to the receptor through the lipid bilayer.

Although the membrane contains several different proteins and is not a very homogeneous surface, the lipid content is very high (19). Therefore, to a first approximation, the vesicle may be considered an isotropic sphere for the purpose of calculating a rate for the first step of the membrane approach model, partitioning of the ligand into the bilayer. The limiting rate for this step was estimated using the Adam and Delbruck (9) relationship for calculating the mean time to collision, τ_3, for spheres diffusing in three dimensions. The Adam and Delbruck model consists of a point ligand and a spherical target (whose radius is equal to the sum of the radii of the ligand and receptor) within a spherical shell of specified size. To apply their model to a realistic situation in which receptors exist in solution at a particular concentration, the total solution volume is viewed as an ensemble of spherical solution volumes, each containing one target molecule. The volume of the spherical solution volumes (cm^3/target molecule) is essentially the reciprocal of the receptor concentration, $[R]$. The radius of this unit volume, c, is

$$c = (4/3 \cdot \pi N_0[R]10^{-3})^{-(1/3)} \quad \text{(A2)}$$

The constants (N_0 is Avogadro's number) in Eq. A2 give c in centimeters. In the limit where the vesicle radius, b, is much smaller than c, the mean time required for the ligand to "locate" the target is

$$\tau_3 = \frac{c^3}{3bD_3} \quad \text{(A3)}$$

In considering the lateral movement within the membrane, the mean time to collision was calculated for circular objects in a plane (or, equivalently, cylinders in a thin slab), using the relationship derived by Weaver (30). In this treatment, it was shown that, for diffusion-driven migration of cell surface components to a circular trap of radius a, the mean time to trapping for a perfectly reactive trap is

$$\tau_2 = \frac{b^2}{D_2}\left(\log\frac{4b^2}{a^2} - 1\right) \quad \text{(A4)}$$

where D_2 is the in-plane diffusion coefficient. The overall time required for binding via the membrane approach, τ_{32}, is the sum of the constituent times:

$$\tau_{32} = \tau_3 + \tau_2 = \frac{c^3}{3bD_3} + \frac{b^2}{D_2}\left(\log\frac{4b^2}{a^2} - 1\right) \quad \text{(A5)}$$

Rate constants corresponding to this τ_{32} are given by $(\tau_{32}[R])^{-1}$.

In order to approach the problem of an anisotropic ligand confined to a plane containing a receptor site, a statistical approach has been used to modify the rates calculated for isotropic ligands. The distinction to be made is that the orientation vector for the ligand must be positioned within θ_0 of a perfect orientation. This geometry, illustrated in Fig. A1, is essentially a two-dimensional analog of the model of Schmitz and Schurr (14). For a ligand with a particular rotational diffusion coefficient, D_R, the probability that the ligand achieves an orientation which results in binding during an encounter of duration τ_E is related to the size of the encounter space (i.e., the region defined by the site radius a) and the rates of lateral and rotational diffusion.

The normal to a line containing the receptor is chosen as the fixed, though arbitrary, reference for the orientation vector. Suppose that the angle between this normal and the orientation vector θ must be $\leq\theta_0$ in order for reaction to occur. For N ligands that reach the encounter space, $N\theta_0/\pi$ will react immediately. Some fraction of the remainder will approach closer to $r = 0$, and others will diffuse to larger r. Individual ligands which diffuse inward from the edge of the encounter space will have encounter times which differ according to the radius of closest approach. The mean duration of these encounters may be estimated by considering the frequency of ligand collisions with a target of radius a and determining the radius of a target which would have half that frequency. Comparing Eq. A4 written for a site of radius a and for a smaller site of radius sa, and specifying $2\tau_2(a) = \tau_2(sa)$, it is easily shown that $s = \sqrt{2.5}a/b$. Since $b \gg a$, the inner radius ($\sqrt{2.5}a^2/b$) is very small, so the mean encounter time will be very nearly that required for a ligand to diffuse a distance $2a$; $\tau_E = 2a^2/D_2$.

For rotation of a cylinder in a slab about an axis

normal to that slab, the mean square angular displacement during time t is $\sqrt{2D_R t}$ (28). Therefore, of the $N(1 - \theta_0/\pi)$ ligands which do not react immediately, $N(1 - \theta_0/\pi)(\sqrt{2D_R\tau_E}/\pi)$ will undergo sufficient rotation during an encounter to react. Therefore, the net probability of reaction is

$$P_2 = \frac{\theta}{\pi} + \left(1 - \frac{\theta}{\pi}\right)\frac{\sqrt{2D_R}}{\pi}\frac{2a^2}{D_2} \qquad \text{(A6)}$$

τ_2/P_2 replaces τ_2 in Eq. A5. This treatment ignores the effects of multiple collisions which occur because the ligand at a is spatially correlated to the target site, an observation first presented for three-dimensional diffusion by Berg and Purcell (10). In two dimensions, the mean number of collisions of a particle with a target, before that particle reaches some equilibrium (uncorrelated) displacement, may be obtained using a relationship derived by Buas (35). For $\bar{n}$ collisions, the probability of a successful collision becomes $1 - (1 - P)^{\bar{n}}$. The effect of this consideration is presented in Discussion.

GLOSSARY

- a: radius of a target site
- b: radius of a vesicle containing a receptor
- c: radius of a spherical volume of solution containing one receptor-containing vesicle
- D_2: diffusion coefficient of a ligand constrained to a membrane
- D_3: diffusion coefficient of a ligand in bulk solution
- D: rotational diffusion coefficient of a ligand
- k_3: forward rate constant for the aqueous approach
- k_{32}: forward rate constant for the membrane approach
- l: order index for Legendre polynomials
- $[L]$: molar concentration of ligand
- n: number of ligand/receptor collisions
- N: number of ligands that reach an encounter space
- N_0: Avogadro's number
- P_l: lth Legendre polynomial
- P_2: probability of reaction for an asymmetric ligand in a membrane
- r: distance from a ligand to its receptor
- r_L: radius of a ligand
- $[R]$: molar concentration of receptor sites
- θ: angle between a ligand orientation vector and the bilayer normal in two dimensions
- θ': angle between a ligand orientation vector and the bilayer normal in three dimensions
- θ_0: angle defining reactive fraction of a ligand in two dimensions
- θ_0': angle defining reactive fraction of a ligand in three dimensions
- τ_2: mean time to ligand/receptor collision in two-dimensional diffusion
- τ_3: mean time to ligand/receptor collision in three-dimensional diffusion
- τ_3': mean time to ligand/vesicle collision in three-dimensional diffusion
- τ_{32}: net mean time to ligand/receptor collision for membrane pathway

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Send reprint requests to: David G. Rhodes, Department of Medicine, University of Connecticut Health Center, Farmington, CT 06032.