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Equilibrium and Kinetic Studies of the Interactions of Salmeterol with Membrane Bilayers

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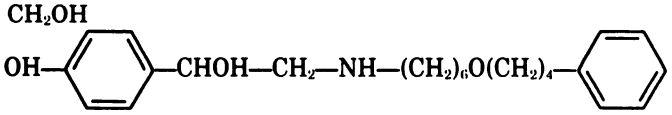
SUMMARY

The interaction of salmeterol with model membranes has been studied with regard to equilibrium and kinetic behavior, including determination of the membrane-based partition coefficient, the rate of dissociation of salmeterol from membranes, and the rate of association. These data were obtained in various membrane preparations and under various conditions (e.g., temperature, cholesterol content). The compound is very lipophilic, compared with other β_2 agonists such as salbutamol, and has a rapid association rate and a moderate dissociation rate. The equilibrium data support the assertion that the salmeterol action measured in perfused tissue involves an *exo*-site for nonspecific binding that may be identified with or related to the lipid bilayer. The kinetic data in unilamellar and multilamellar liposomes of synthetic lipids further suggest that the approach to the *exo*-site and the active site may involve components in the native system other than the lipid bilayer in which the β_2 receptor is located. These additional components may explain the slow onset and the extraordinarily long duration of action.

Salmeterol (**1**) is a long acting β_2 agonist that resembles salbutamol with an extended aliphatic domain.



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Work by others with perfused guinea pig trachea has shown that this compound has a relatively slow onset but, once active, exerts its effect for a very long time (>15 hr) (1). It has been suggested (2) that salmeterol interacts with an *exo*-site near the β_2 receptor that weakly binds the drug and accounts for the long duration of action. The activity of salmeterol could be blocked in perfused guinea pig trachea by sotalol but, upon flushing with buffer, activity returned. The blocking and rinsing cycle could be carried out repeatedly, with salmeterol activity consistently returning to pre-block levels. In perfusion systems (1) salmeterol has a long onset time, 17 min, compared with 1-2 min for salbutamol or formoterol. Because the octanol/H_2O partition coefficient is so high, some (3) have suggested that the *exo*-site may be the membrane bilayer itself, but others (4) have suggested that the nonpolar substituent interacts with nonpolar amino acid residues in the β_2 receptor protein. Because the structure of salmeterol appears to be amphiphilic, and its long residual time suggested that membrane bilayer partitioning might be involved in the overall binding mechanism, an investigation of the interaction of salmeterol with membrane bilayers was undertaken.

In previous work with a variety of other drugs (e.g., 1,4-dihydropyridine calcium channel agonists and antagonists), it was demonstrated that these drugs interact with lipid bilayers by partitioning to a well defined location in what may be the first step leading to a high affinity interaction with their receptor (5, 6). This so-called 'nonspecific' interaction of these drugs with membranes does, in fact, involve an interaction with a very specific structural basis. Although the lipid bilayer is a dynamic fluid structure, there are structural features that provide stable unique domains in which very specific chemical interactions can occur. A model has been proposed for 1,4-dihydropyridine binding to membrane-associated receptors (Ca^{2+} channels) in which the drug first partitions into the lipid bilayer and then diffuses laterally to approach the active site on the receptor protein (7). We have shown, using X-ray and neutron scattering, that when this nonspecific partitioning into the lipid bilayer occurs the drug molecules adopt a well defined equilibrium location (depth in the bilayer). For many of the compounds we have investigated, this location has been shown to be at the interfacial region between the hydrophilic headgroup and the hydrophobic core.

In addition, it is likely that the drug also adopts a specific orientation in this asymmetric environment. Although this

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ABBREVIATIONS: DOPC, dioleoylphosphatidylcholine; CH, cholesterol.

orientation is expected for amphiphilic molecules like propranolol (8), it is possible that other molecules have some preferential orientation as well (5). Because the membrane environment is so markedly different from the isotropic environment of free solution or the tightly packed crystal structure, the conformation of the membrane-associated drug is likely to be unique. If the drug approaches its binding site via the lipid bilayer, this structure is significant because it is the conformation that the drug has in the vicinity of the receptor site that will ultimately interact with that site.

Materials and Methods

Salmeterol, salbutamol, and labeled analogs were synthesized at Glaxo Group Research. DOPC and other lipids were purchased from Avanti Polar Lipids (Alabaster, AL), checked for purity using thin layer chromatography, and used without further purification. CH was purchased from Avanti and used without further purification. [^{3}H]CH and [^{14}C]DOPC were purchased from New England Nuclear. Other reagents (buffer components and other salts) were purchased from Sigma and Aldrich and were used without further purification. Water was purified using a Barnstead RGW-5 ultrafiltration system, which produces reagent-grade (18 MΩ) water.

Multilamellar liposomes (Fig. 1A) were prepared by shell-drying lipids from a $CHCl_3$ solution in a test tube, adding buffer, and mixing vigorously with a vortex mixer for 3 min. These liposomes were typically a heterogeneous population of 100–500 nm in diameter, according to electron micrographs. Unilamellar liposomes (Fig. 1B) were produced by repeatedly extruding (15 times) the multilamellar preparation through a 100-nm filter (Liposofast, MM Developments, Toronto, Canada). The liposomes were typically 100 nm in diameter and appeared to be unilamellar in electron micrographs.

The membrane partition coefficient, $K_{p[mem]}$, was determined using previously reported methods (6). Briefly, [^{14}C]salmeterol (100 nM) was equilibrated with membrane preparations (20 μg/ml) for 30 min at 25° (or other desired temperatures). Equivalent control samples were prepared containing 100 nM drug only. Aliquots (5 ml) were filtered through Whatman GF/C glass fiber filters on a Brandel M-48R cell harvester. Association rates were determined by adding the labeled salmeterol to the liposome suspensions at various times before filtration.

The amount of membrane associated with the filter was determined by establishing a retention efficiency using [^{14}C]DOPC- or [^{3}H]CH-labeled liposomes. Data were corrected for retention, which was 80% for multilamellar liposomes and 62% for unilamellar liposomes. Retention did not depend on CH concentration over the range of CH concentrations used in this work. The amount of drug remaining in aqueous solution at equilibrium was determined by subtracting the amount of bound drug from the initial aqueous concentration. The membrane-based partition coefficient was calculated as:

$$K_{p[mem]} = \frac{g(\text{drug})/g(\text{lipid})}{g(\text{drug})/g(\text{water})}$$

where the weight of drug is determined from D_m (the amount of membrane-associated drug), the weight of lipid from the amount in the reaction mixture and the measured retention, and the denominator as a weight ratio expression of the aqueous concentration after correction for partitioned drug. $K_{p[mem]}$ is referred to as a weight-based number so that data in heterogeneous biological membranes may be easily defined and because localization of drugs to specific domains in lipid bilayers obscures the meaning of the molar concentration of a partitioned compound (see below).

Dissociation kinetics were determined by measuring the amount of drug retained on filters in a large volume of buffer as a function of time. Filter circles with membrane and drug associated and those with drug only (prepared as described above) were impaled on stainless steel needles attached to a Plexiglass disc, which fit near the base of a 1-liter beaker. The beaker, typically containing 10 filters, was placed on a stirring plate at low speed and buffer (10 mM Tris, 150 mM NaCl, pH 7.0) was added at $t = 0$. Of the liposomes retained on the filter during initial filtration (no rinsing), 90% were tightly bound and did not dissociate from the filter, even after 15 hr. The unbound 10% released quickly (<1 min) and probably represent trapped unbound liposomes. The unbound fraction did not depend on whether the liposomes were unilamellar or multilamellar. At desired time intervals, filters were removed from the needles and placed in scintillation vials for counting (Searle Delta 300 6890 LSC). Counts from filters containing drug alone were subtracted from corresponding data from samples that contained membrane and drug (drug being associated both with filters and with membrane). The more rapid loss of counts from control filters was interpreted as loss of filter-associated drug (D_f). This process could be observed as an early phase of dissociation from filters with liposome-

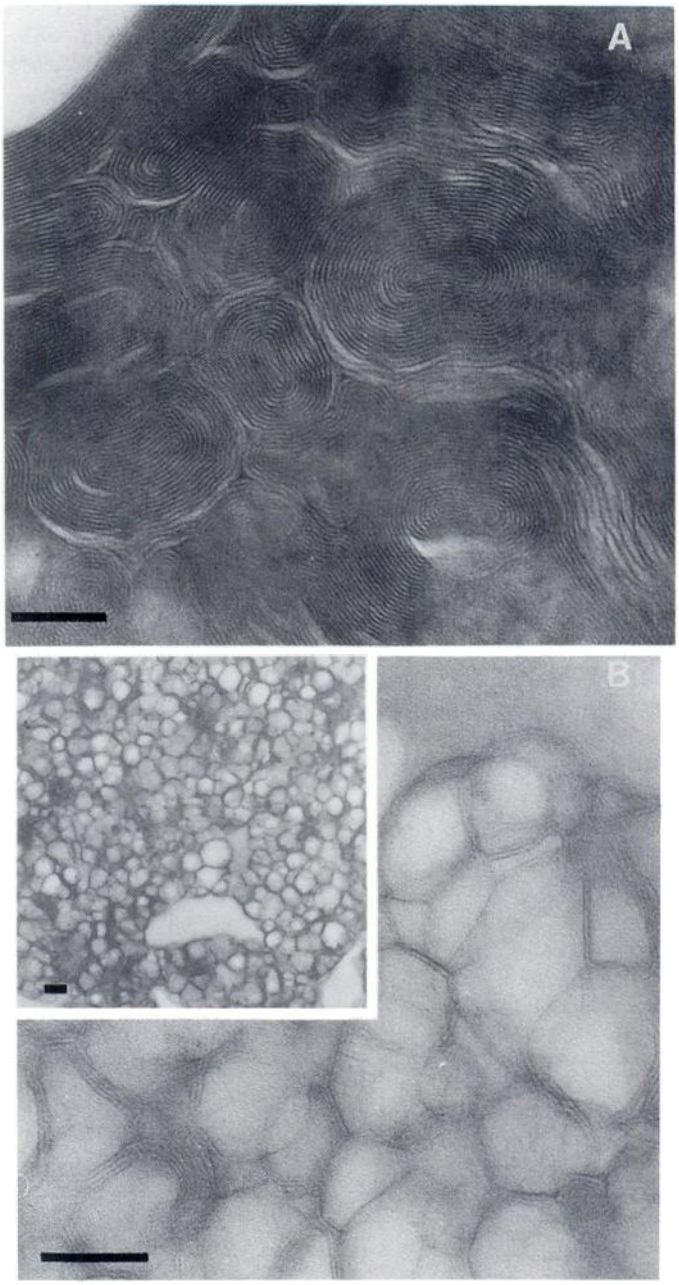

Fig. 1. Multilamellar (A) and unilamellar (B) preparations of liposomes used in this study. *B, Inset* shows a wider field (low magnification) view of the unilamellar preparation. *Scale bars*, 100 nm.

associated drug, but these also exhibited a slower loss of membrane-associated drug (D_m). Although D_m could not be directly determined at zero time, unrinsed filters obtained under identical conditions provided $D_m + D_f$, and extrapolation of the dissociation curve to zero time yielded an estimate of D_m.

Because the amount of drug associated with the filters decreased with time, the relative amount of drug could be expressed as an apparent $K_{p[mem]}$, $K'_{p[mem]}$, rather than in terms of counts, in order to account for other experimental variables (total concentrations of drug and membrane). The extrapolated $K_{p[mem]}$ ($K'_{p[mem]}$ at $t = 0$) could be estimated to ~10%. The dissociation kinetics were not modeled in terms of a specific dissociation mechanism. Rather, the data described above were used to determine $\tau_{1/2}$, the time required for $K'_{p[mem]}$ to reach half the original ($t = 0$) value. The data appeared to follow an exponential decay.

To consider $K_{p[mem]}$ as a true association constant, one must express the partitioning in terms of molar concentrations. The difficulty arises when one attempts to convert mass ratio-based concentrations of membrane-associated drug to molar concentrations. Because membrane-associated drugs tend to be localized (9),[1] it is not appropriate to use the entire membrane volume (surface area × thickness) as the volume in which the drug is dispersed. Similarly, because the drug is of finite size and is probably distributed about a mean location according to an energy distribution function, the volume is finite but ill defined. It was because of this problem, and to allow direct comparison of data from model systems (in which the molecular weights of the lipid 'solvent' molecules are known) with data from biological systems (which have heterogeneous lipid populations), that the mass ratio system was originally adopted for use in this laboratory (6).

As a simple approximation for the present case, one could consider that most of the salmeterol molecule is hydrophobic and that the length of the molecule may occupy most of the membrane bilayer interior. If the hydrophobic interior of the bilayer is ~35 Å thick and each lipid molecule occupies 60 Å^2 of area, the hydrophobic volume of each lipid is 1050 Å^3. Using the molecular weights of DOPC and water, one may easily show that the conversion from [g(drug)/g(lipid)]/[g(drug)/g(buffer)] to c(membrane)/c(buffer), where c refers to molar concentration, involves a factor of 1.24. (The molecular weight of the drug is eliminated from the fraction.) It is interesting to note that the mass ratio $K_{p[mem]}$ and the molar $K''_{p[mem]}$ are numerically equal if the occupied membrane thickness is assumed to be ~44 Å. At the other extreme, if a small drug is localized to a thin slab ~4.4 Å thick, conversion from the mass-based $K_{p[mem]}$ to the molar $K''_{p[mem]}$ would involve a factor of 10.

Results

The bulk partitioning (i.e., octanol/H_2O) behavior of salmeterol was not measured, because this has already been determined for salmeterol and would not be likely to correlate with the membrane-based partition coefficient $K_{p[mem]}$ (6). Isotropic octanol/H_2O K_p and membrane-based $K_{p[mem]}$ have been determined in this laboratory for a number of compounds (e.g., see Table 1), and no correlation has been found between the two parameters.

Although salmeterol persistence had been demonstrated in isolated guinea pig trachea preparations, the present work was with model lipid bilayers because of the limited availability of trachea lipids. It has been shown (10) that for many drugs the equilibrium $K_{p[mem]}$ depends strongly on the composition of the membrane and its physical state. For example, CH added to a membrane in a ratio of 1:2 can decrease the $K_{p[mem]}$ for certain drugs by 50%. For lipid bilayers composed of synthetic lipids, reducing the temperature to a point below the phase transition temperature can reduce $K_{p[mem]}$ from values of ~10^3 to unmeasurably small values. Because the composition of biological membranes varies widely, one might argue that the only appropriate test medium is the 'host' biological membrane itself. Empirically, however, it has been found that many biological membranes can be reasonably simulated by appropriate proportions of DOPC and CH, usually with CH at approximately the proportion found in the biological membrane (10). Because the CH content of the membrane surrounding the β_2 receptor has not been verified, the composition of the membrane was approximated by a 'moderate' CH to DOPC ratio of 0.3:1 (see below).

TABLE 1

Partition coefficients and dissociation τ½ values

The data for salmeterol were obtained with unilamellar liposomes; the data for all other drugs were obtained with multilamellar liposomes of the same composition. Comparison is still valid, because there is no evidence of any dependence on this factor in any of the other systems. The data for amiodarone were obtained in lipid mixtures extracted from sarcoplasmic reticulum. $K_{p[mem]}$ is likely to be similar to that for amiodarone in DOPC/CH, because $K_{p[mem]}$ values of the other drugs have also been determined in sarcoplasmic reticulum lipids, and the data are similar.

Drug	$\tau_{½}$	$K_{p[mem]}$	$K_{p[o/w]}$[a]
	min		
Nimodipine	<5	2,700	260
Amlodipine	100	21,800	30
Salmeterol	60	22,500	7,600
Amiodarone	1,000	~1,000,000	350

[a] $K_{p[o/w]}$, octanol/water partition coefficient.

The association rate was determined by observing relative amounts of bound drug as a function of CH concentration. Regardless of membrane composition, the association rate was quite rapid (Fig. 2). $K_{p[mem]}$ determined as a function of time did not change from 1 min (shortest measurable time) to 1200 min. This rate was independent of whether the liposomes used were multilamellar or unilamellar. That is, the drug rapidly equilibrated with all of the lipid population with which it ultimately interacted. However, the final proportion of associated drug was significantly higher for unilamellar preparations (see below).

Although association rates were not affected by CH, it was found that CH reduced $K_{p[mem]}$ (Fig. 3), a result that is consistent with data for other drugs (10). The dependence of $K_{p[mem]}$ on CH was only moderate, however, changing $K_{p[mem]}$ by only a factor of 3 over the full range of CH concentrations. To approximate the physiological system, therefore, a moderate concentration of 0.3:1 CH/DOPC (typical for a variety of biological membranes) was used for many subsequent experiments.

A significant difference was observed in $K_{p[mem]}$ measured in unilamellar and multilamellar liposomes. Membrane partition coefficients were determined for multilamellar and unilamellar liposomes with compositions varying from DOPC to 0.6:1 CH/DOPC, with drug being added to preformed liposomes. The data were corrected using the retention values specific to unilamellar and multilamellar liposomes. These data indicated that $K_{p[mem]}$ for the unilamellar preparations of a particular composition was always higher than for multilamellar preparations of the same composition. On average, the $K_{p[mem]}$ of unilamellar liposomes was 1.4 times higher than that of the multilamellar liposomes. These data suggested that there may not be complete equilibration of salmeterol with the interior lamellae of the multilamellar liposomes.

This conclusion was supported by experiments in which

[1] D. G. Rhodes et al., unpublished observations.

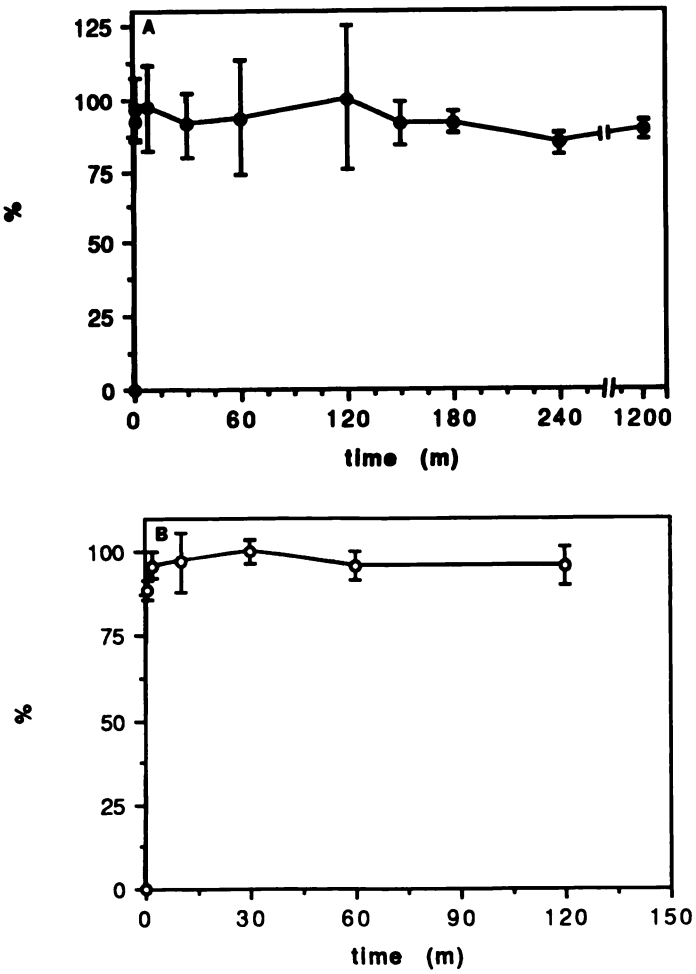

Fig. 2. Association of salmeterol with multilamellar (A) and unilamellar (B) liposomes. The composition of the liposomes in each case was 0.3:1 CH/DOPC. Similar experiments at different temperatures and CH contents yielded similar results.

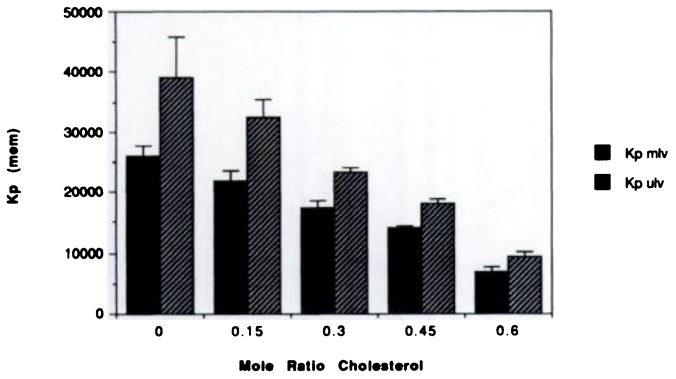

Fig. 3. Dependence of $K_{p[mem]}$ on composition in DOPC/CH liposomes. Measured $K_{p[mem]}$ was always lower in multilamellar preparations (■) than in unilamellar preparations (▨). Although the composition dependence was strong, salmeterol remained very lipophilic throughout the range of CH concentrations used. Even at 0.6:1 CH/DOPC, the $K_{p[mem]}$ of salmeterol was comparable to that of compounds known to be membrane active.

salmeterol was forced into the interior lamellae by addition of the drug in the organic phase. [^{14}C]Salmeterol, DOPC, and (for some experiments) CH were mixed in $CHCl_3$ solution, shell-dried together to a film, as before, and hydrated to form multilamellar liposomes with incorporated [^{14}C]salmeterol. This procedure ensured complete equilibration of salmeterol with the entire lipid population. Based on the concentration of salmeterol and the average size of the liposomes, one would not expect significant amounts of passively trapped salmeterol (<4 molecules/liposome). This preparation resulted in an apparent $K_{p[mem]}$ value approximately twice that of preparations in which [^{14}C]salmeterol was added to preformed vesicles. These results strongly suggest that salmeterol does not penetrate beyond the first layer of a multilamellar system (see Discussion).

Dissociation rates were determined by measuring dissociation of labeled drug from filters with retained liposomes with partitioned drug. Filters with free salmeterol only or with membrane/salmeterol and free salmeterol were sampled in pairs as a function of time. The free salmeterol washed out quickly, reducing the activity of the drug-only control filters to near zero within 15 min ($\tau_{1/2}$, ~5 min) (Fig. 4). The filters with membranes and salmeterol showed an apparently biphasic dissociation, with a small rapid phase believed to correspond to dissociation of free drug and a slower dissociation presumably due to dissociation of drug from liposomes. The dissociation from liposomes was calculated by correcting for the filter-associated drug, based on dissociation rates from the control filters without liposomes. The apparent membrane partition coefficient $K'_{p[mem]}$ was calculated as a function of time. By

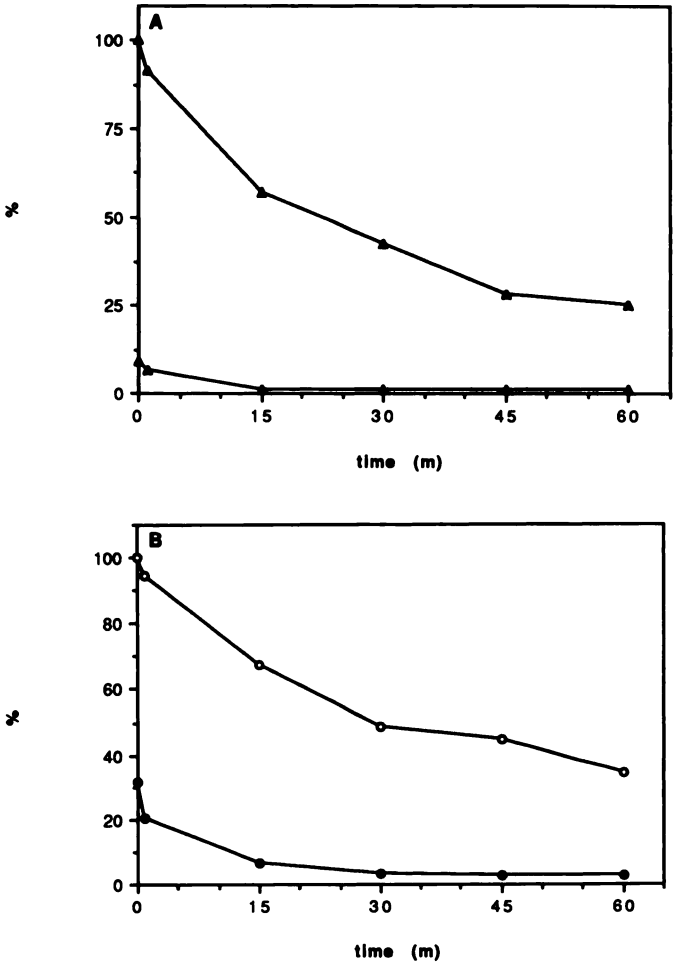

Fig. 4. Dissociation of salmeterol from multilamellar (A) and unilamellar (B) liposomes. [^{14}C]Salmeterol was added at an initial total concentration of 100 nM. In both A and B, the *lower curve* (▲, ●) is from filters with drug only and the *upper curve* (△, ○) is from liposome-containing filters.

extrapolation, then, $K_{p[mem]}$ ($K'_{p[mem]}$ at $t = 0$) could be determined.

Although D_f estimated with unrinsed filters corresponded to D_f extrapolated from the dissociation curves, D_m estimated from dissociation curves was always higher than D_m estimated directly from unrinsed filters. There appeared to be a brief increase in the amount of partitioned drug on the filter. (Note that the total amount of drug decreased monotonically but the amount of partitioned drug after correction for filter-associated drug briefly increased.) Although the explanation has not yet been proven directly, it is likely that some of the filter-associated drug was partitioning into filter-associated liposomes. Making a few simple geometric assumptions to estimate the filter volume, and neglecting the volume of the filter fiber itself, it may be estimated that the filter-associated salmeterol is present at ~3 times the aqueous concentration. Rinsing the drug from the filter caused a briefly elevated concentration of salmeterol, some of which partitioned into the filter-associated liposomes. This explanation is supported by the fact that the effect was most pronounced in experiments with unilamellar liposomes (Fig. 5), in which, for an equivalent mass of lipid, the surface coverage of the filter would be more complete, even at reduced retention ratios.

Using the elevated data for membrane-associated salmeterol, $\tau_{1/2}$, the time required to dissociate half of the drug from the liposomes into solution, was measured at several temperatures with multilamellar and unilamellar liposomes. $\tau_{1/2}$ was approximately 25 min, but the dissociation was temperature dependent, being accelerated at increased temperatures (Fig. 6). However, the rate of dissociation was equivalent from unilamellar and multilamellar liposomes if drug was added to preformed liposomes.

For salmeterol trapped in multilamellar liposomes as described above, the dissociation was significantly slower, with $\tau_{1/2} \simeq 180$ min (Fig. 7). Although this supports the hypothesis that salmeterol does not penetrate bilayers, the fact that any significant dissociation was observed calls this conclusion into question. It should be kept in mind, however, that the multilamellar preparations appear in electron micrographs (Fig. 1A) to be heterogeneous and irregular and may have structural defects (leaks). Further, the process of binding the liposomes

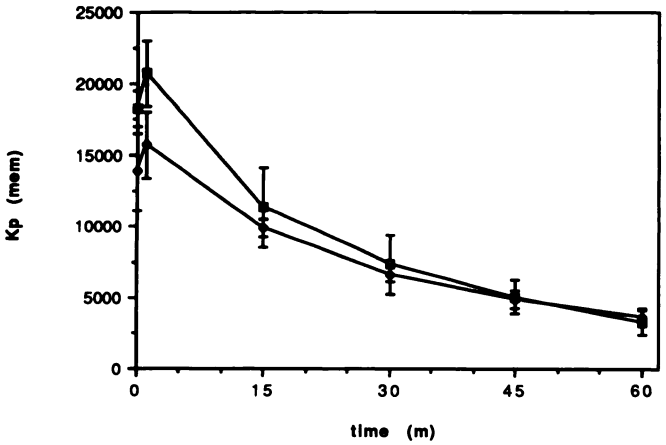

Fig. 5. Corrected dissociation of salmeterol from unilamellar (⊡) and multilamellar (♦) liposomes. At very early times, there was an apparent increase in the $K_{p[mem]}$ due to the partitioning of filter-associated salmeterol. This effect was generally more pronounced for unilamellar preparations.

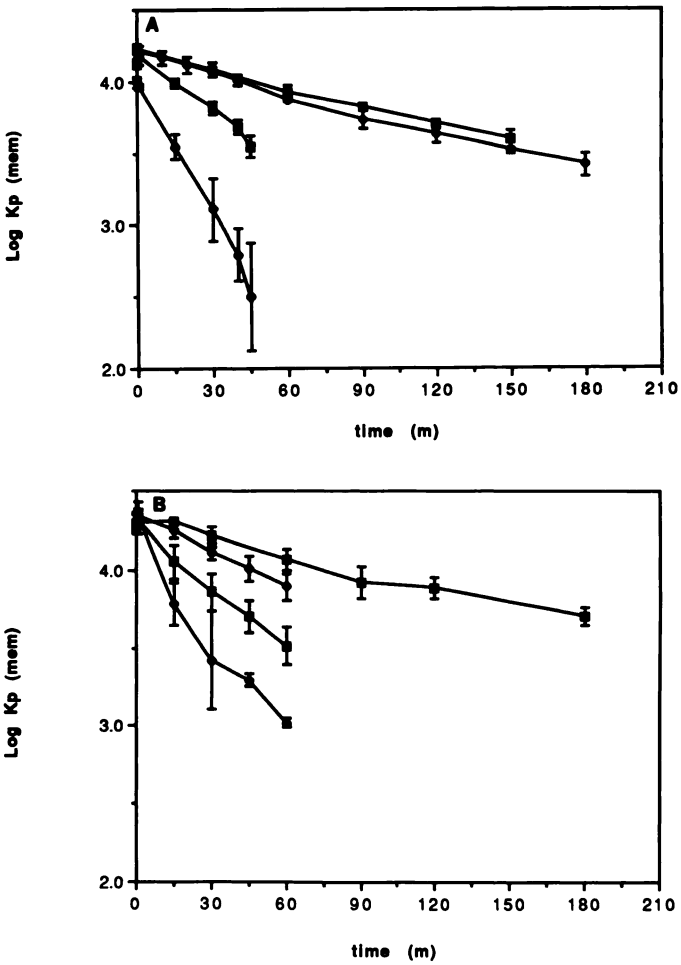

Fig. 6. Temperature dependence of salmeterol dissociation from multilamellar (A) and unilamellar (B) liposomes. ⊡, 15°; ♦, 25°; ■, 37°; ◇, 55°.

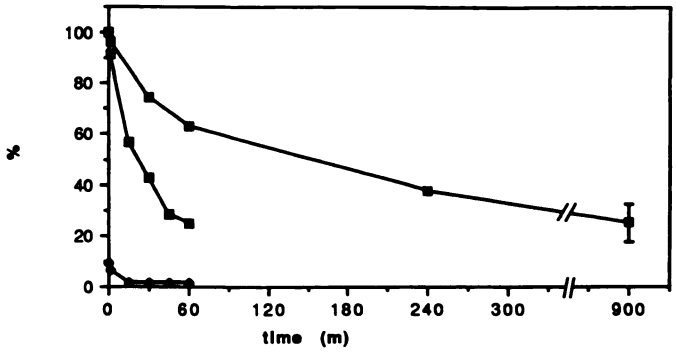

Fig. 7. Dissociation of salmeterol from multilamellar liposomes in which drug had been incorporated from the solvent phase. The data at the *left side* are the same as in Fig. 4A. $\tau_{1/2}$ was approximately 180 min, and the time required to reach 20% of initial value was 15 times that of preparations to which the drug had been added externally.

to filters may induce such leaks. This conclusion is supported by the fact that the apparent $K_{p[mem]}$ for liposomes with trapped salmeterol did not depend on incubation time. That is, the salmeterol associated with multilamellar liposomes in solution, without addition of labeled drug to the aqueous phase, was essentially constant whether the liposomes were filtered immediately after preparation or hours later.

At 25° and 0.3:1 CH/DOPC, $K_{p[mem]}$ is 22,500. (This value is

in terms of [g(drug)/g(lipid)]/[g(drug)/g(H_2O)] and is for unilamellar liposomes.) By way of comparison, the isotropic partition coefficient (octanol/H_2O) is 7600. By comparison with other membrane active drugs, this $K_{p[mem]}$ value is comparable to that of very lipophilic compounds like amlodipine but not as high as that of as amiodarone. These two compounds have very slow rates of dissociation from membranes. Dissociation of amiodarone ($K_{p[mem]}$ ~10^6) is characterized by a $\tau_{1/2}$ of ~10^3 min. The $\tau_{1/2}$ for amlodipine ($K_{p[mem]}$ = 19,000) is 10^2 min. Nimodipine ($K_{p[mem]}$ = 6000) has dissociation kinetics more typical for lipophilic drugs, with $\tau_{1/2}$ of ~5 min. Salmeterol has a $\tau_{1/2}$ of 25 min at 25°. This is comparable to the $\tau_{1/2}$ for amlodipine but is much smaller than one would expect based on data from salmeterol perfusion studies.

It is also interesting to compare data for salmeterol with corresponding data for salbutamol. In experiments with labeled salbutamol in multilamellar liposomes, no significant partitioning into membranes could be demonstrated. Limits could be established using the known membrane and drug concentrations and the detection limits of the protocol. Under the same conditions of temperature and membrane composition in which $K_{p[mem]}$ for salmeterol is measured to be 22,500, $K_{p[mem]}$ for salbutamol is ≤4.

Discussion

Although salmeterol is now used clinically, the mechanism of its long duration is not well understood. It has been shown for a number of other drugs (5, 6) that long duration and perhaps increased apparent association constants can result from the so-called nonspecific interaction of these drugs with the membrane lipid bilayer. In particular, it has been shown that a number of drugs that associate with lipid bilayers adopt a very specific position in the bilayer, often at the interface between the hydrophobic interior and the aqueous headgroup region. This has been observed not only for obviously amphiphilic drugs (8) but also for a number of drugs that do not appear to be strongly amphiphilic. This partitioning not only may concentrate the drug in the membrane but also may position and orient the drug so as to facilitate specific binding at the membrane-associated receptor protein.

The issue addressed in this work was whether the *in vivo* or *in vitro* kinetic profile of salmeterol could be explained by its interaction with the lipid bilayer, specifically in light of its extraordinary duration and its ability to remain near the active site (perhaps at an *exo*-site) even with extensive rinsing in the presence of antagonist. It has been suggested (4) that salmeterol remains in the vicinity of the active site by virtue of its association with the lipid bilayer. This work was intended to address this possibility. Although the onset time for salmeterol is ~17 min in perfused guinea pig trachea preparations, the partitioning of salmeterol into the lipid bilayer was complete within 1 min in model membrane systems. The long delay in the case of the intact tissue could be due to differences in the ultrastructure. The drug must reach not just the lipid membrane at the surface of the airway interior but the membrane that contains the β_2 receptor. In the results presented here for model lipid bilayers, it was demonstrated that association was complete within <1 min with unilamellar or multilamellar liposomes and that dissociation was much more rapid than observed in perfusion studies. However, the equilibrium $K_{p[mem]}$ was significantly larger for the unilamellar liposomes, indicating that salmeterol could not easily reach 'interior' lamellae and does not traverse the outer lamellae to any significant extent.

The data for multilamellar liposomes presented in Fig. 2A suggest that an even more stringent constraint may be stated. The drug molecules in solution have immediate access only to the outer monolayer of the surface bilayer. If salmeterol were able to "flip-flop" from the exterior surface monolayer to the interior monolayer of the outer bilayer, one would expect that some dissociation to the first inter-bilayer water space would be possible. The dissociation data from the unilamellar liposomes (Fig. 4B) provide a time-course for such dissociation. This would allow a slow equilibration of drug with all of the lipid bilayers in the multilamellar system. Because the equilibrium $K_{p[mem]}$ is established in <1 min and is stable for many hours, it is probable that the salmeterol molecules do not even pass the outer monolayer of the surface bilayer. It should be emphasized here that a kinetic explanation for the partitioning data, although unlikely, is based primarily on the long (20 hr) stability of the association curve (Fig. 2A). Dissociation of salmeterol trapped within multilamellar liposomes was significantly slower than that from liposomes to which salmeterol had been added externally, but some dissociation did occur. Although it is likely that this resulted from physical disruption of the liposomes on the glass fiber filter, transbilayer diffusion cannot be excluded from these data alone.

This result with lipid bilayers suggests a model in which salmeterol must traverse certain membrane barriers *in vivo* and this accounts for the observed long onset of activity. For example, the clinical onset time might be accounted for by assuming association with an exposed surface of an epithelial cell, diffusion to the opposite face, dissociation from the epithelial cell, association with the membrane containing the β_2 receptor, and binding to the receptor. Because the membrane and receptor association steps would be very rapid (seconds) and the lateral diffusion step moderately rapid (minutes), the rate-limiting step would be the dissociation from the epithelial cell membrane ($\tau_{1/2}$ ~25 min). The long duration of action could involve 'trapping' of the salmeterol in the vicinity of the membrane with the β_2 receptor. This explanation does not address the perfusion data, in which barrier layers would not necessarily be intact, but other similar explanations could be proposed. Clearly there are many intermediate steps that would have to be demonstrated, but such approaches are possible. Regardless of such details yet to be discerned, it is apparent that the lipid bilayer component is involved in salmeterol binding to its receptor.

To summarize then, salmeterol is a highly lipophilic compound, with its $K_{p[mem]}$ being among the highest measured in this laboratory, whereas salbutamol has a very low $K_{p[mem]}$ of <4. Salmeterol does not appear to penetrate the outer bilayer of multilamellar systems and does not interact with the interior lamellae. Salmeterol rapidly associates with model lipid bilayers to which it has access but dissociates slowly. Although slow, this rate of dissociation from lipid bilayers is much faster than the rate of disappearance of β_2 activity in perfused systems. Similarly, the rate of association with a lipid bilayer is much faster than the rate of onset of β_2 activity in perfused systems. Thus, although the lipid bilayer is probably involved in the approach of salmeterol to its active site and may be related to

the *exo*-site, the overall mechanism is likely to involve other components, possibly related to cellular architecture.

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