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Comparisons of the Interaction of Propranolol and Timolol with Model and Biological Membrane Systems

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SUMMARY

The nonspecific interaction of the *beta*-adrenergic blocking drugs, propranolol and timolol, with model and biological membranes has been investigated. Radioisotope measurements of the association of these drugs with dimyristoyl lecithin (DMPC) bilayers showed that both propranolol and timolol had a significantly greater molar association (mole of drug per mole of lipid) with DMPC above its phase transition temperature than below. Timolol had a much lower molar association with DMPC as compared with propranolol both above and below the phase transition temperature. For the DMPC model membrane system, the molar association of propranolol as measured by radioisotope and inferred from calorimetric studies was similar. Neutron diffraction utilizing propranolol deuterated in the naphthalene moiety showed that the naphthalene moiety of propranolol partitions into the hydrocarbon core of the DMPC lipid bilayer, and that the charged amine side chain is most likely positioned in the aqueous phospholipid head group region. For timolol, the association as measured by radioisotope methods was apparently greater than the partitioning inferred from calorimetric studies using freezing point depression analysis, suggesting a more complex interaction of timolol as compared with propranolol with the DMPC lipid bilayer. The association of propranolol and timolol with sarcoplasmic reticulum vesicles (SR) was similar to that with highly purified protein-depleted SR lipids, and DMPC *above* its phase transition. The association of propranolol with the SR membrane (mole of propranolol per mole of SR phospholipid) correlated with its ability to inhibit calcium uptake, whereas only a fraction of the total association of timolol with the SR membrane appeared to lead to inhibition of calcium uptake. These results suggest that the major *nonspecific* interactions of propranolol and timolol are with the SR membrane lipids, and that the magnitude of their interactions depends on both the lipid solubility of the drug and the physical state of the fatty acyl chains of the membrane. Both propranolol and timolol appear to perturb the functional properties of the calcium pump protein in the SR membrane (inhibition of ATP-induced calcium uptake) indirectly by partitioning into the bulk lipid matrix of the SR lipid bilayer, although other sites of interaction cannot be excluded.

INTRODUCTION

Beta-adrenergic receptor blocking drugs exhibit nonspecific as well as specific membrane effects. The specific interaction with cell membranes, which results in *beta*-adrenergic receptor blockade, is mediated by binding of these drugs directly to sarcolemmal *beta*-adrenergic receptors (1), whereas the nonspecific membrane effects of these drugs appear to involve interactions with structures other than these receptors (2, 3). These nonspecific effects can be manifest in a variety of ways, including inhibition of calcium uptake by both skeletal and cardiac SR[3] vesicles (3). Differences in the inhibitory effects of two *beta*-adrenergic blocking drugs, propranolol and timolol, on calcium uptake by rabbit skeletal SR vesicles probably reflect different nonspecific membrane effects, as calcium transport by these membranes is not regulated by cyclic AMP (4). The ability of these drugs to interact with and cause functional perturbations of other biological membrane systems suggests that their site of non-

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[3] The abbreviations used are: SR, sarcoplasmic reticulum; DMPC, dimyristoyl lecithin; DSC, differential scanning calorimetry.

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specific action may be the lipid component of these membranes (5–8).

Information regarding possible interaction of amphiphilic drug molecules with biological membranes (9), especially the lipid component of these membranes, can be obtained in studies of pure lipid membrane systems, taking into account certain limitations for such systems as models of protein-containing biological membranes (10). Numerous studies, employing techniques such as differential scanning calorimetry and spectroscopic measurements, have defined differences in the structural interactions of a variety of amphiphilic molecules with model membrane systems (11, 12). The interaction of propranolol with model lipid systems has been shown to lower the phase transition, an effect that has been suggested to be related to the functioning of the sodium channel (13). A clearer picture of the interaction of amphiphilic molecules with membranes can be obtained when scanning differential calorimetric and X-ray diffraction measurements are compared (14, 15).

In the present study, the temperature- and drug concentration dependence of the association of radioisotope-labeled propranolol and timolol with model membranes formed from DMPC were investigated. These results were then compared with those obtained by differential scanning calorimetry of the DMPC model membrane system in the absence and presence of the drugs. Association of propranolol and timolol with the native SR membrane and its protein-depleted lipids was also investigated. A model rationalizing the nonspecific membrane effects of propranolol and timolol, and incorporating neutron diffraction data, was formulated in terms of the interactions of these drugs with the membrane bilayer.

MATERIALS AND METHODS

Preparation of biological and model membranes. SR vesicles were isolated by homogenization and differential centrifugation of rabbit hind leg and back white muscle by a modification (16) of the method of Harigaya and Schwartz (17). The vesicles were purified further by isopycnic sucrose density gradient centrifugation, yielding a population of functional, closed, unilamellar membranous vesicles of "light" SR [115 moles of phospholipid per mole of calcium pump protein (18)]. Various concentrations of propranolol and timolol were added to light SR vesicles in a buffer medium containing 100 mM KCl, 10 mM $MgCl_2$, and 10 mM Tris maleate (pH 7.0).

In some experiments, the SR lipids (greater than 95% phospholipids) were extracted from light SR with chloroform/methanol (2:1) (18), after which propranolol or timolol was added to the solubilized lipid extract and heated to 45° under a stream of nitrogen. For radioisotope studies, [^{3}H]propranolol or timolol was added as the tracer prior to heating. The dried sample was then placed under vacuum at 45° for 2 hr, after which time the buffered medium (100 mM KCl, 10 mM $MgCl_2$, and 10 mM Tris maleate, pH 7.0) was added at 20°. The sample was vortexed, allowed to equilibrate for 15–30 min, and used in the studies described below. This procedure yields multilamellar lipid structures as seen in the electron microscope (Hitachi H300) in thin section.

In studies of model membranes,[4] synthetic DMPC was added to chloroform. Propranolol or timolol dissolved in chloroform/methanol (2:1) was then added to the lipid solution, and the procedure described above for SR lipids was utilized except that the buffer contained only 20 mM Tris (pH 7.5), yielding multilamellar lipid dispersions for both radioisotope and calorimetric studies. These dispersions were subsequently equilibrated at various temperatures between 0° and 45° and used in the studies described below.

The concentrations of drugs reported throughout this paper refer to those in the aqueous phase immediately after drug addition and not to final concentrations in the aqueous phase after equilibration with membranes. The molar ratios for the drug-lipid system were obtained at equilibrium, since these values were independent of equilibration time over a period of hours using the above-described procedure.

Radioisotope measurements of the association of propranolol and timolol with membranes. The association of propranolol and timolol with the DMPC model and isolated SR membranes was quantified using ^{3}H-labeled propranolol or timolol. We use the term "association" for radioisotope measurements since these studies provide no information regarding the localization of the drug molecules in the membrane. Various concentrations of each drug were added to the model and SR membrane preparations described above. The samples were allowed to equilibrate at various temperatures between 0° and 45° and subsequently centrifuged at 100,000 × *g* in microcentrifuge tubes (400 μl) for 1 hr in a Beckman SW 27 swinging bucket rotor at the same temperature at which they had been equilibrated. Aliquots (50 μl) of the supernatant were sampled before and after centrifugation and added to a Biofluor scintillation mixture. The pellets were cut from the centrifuge tubes and carefully blotted dry, after which they were added to Biofluor and counted for ^{3}H radioactivity. The mixing of the drug and lipid prior to the formation of multilamellar lipid structures by vortexing in buffered medium was found to be essential, since the addition of drugs to preformed lipid dispersions consistently yielded lower drug-lipid association values that were a function of incubation time. Equilibration of this drug-lipid system was relatively slow and probably the result of rate-limiting diffusion of drugs into the multilamellar lipid structures. The above procedure in which drug and lipid were co-vortexed yielded association values that were independent of equilibration time.

In control experiments, ^3H_2O was used to measure the water space of the packed pellet. The water content of the packed DMPC pellets below the phase transition was twice that above the phase transition but was not affected by the presence of either drug both above and below the phase transition. In all cases, the pellet water content exceeded the maximal hydration of DMPC as reported by Janiak *et al.* (19), indicating either that an excess of interliposomal water phase was trapped in the pellets or that adsorbed molecules (such as Tris, or free fatty acid impurities) increased the interbilayer space beyond the maximum observed by Janiak *et al.* (19). The correction for the drugs trapped in the water space of the packed pellet would amount to less than 5–10%[5] when the partitioning of the drugs into the lipid is taken into account (see below). Thus, the *total* association of propranolol or timolol with the membranes is expressed in this paper as moles of drug per mole of phospholipid without taking into account the water space

[4] The procedure described for preparation of the drug-model liposomal system was chosen because of the difficulty in preparing large (>500 Å) unilamellar pure lipid vesicles, which is the ideal system required for such a study. Use of small unilamellar lipid vesicles (250–500 Å) can cause problems in measuring amphiphile-lipid interactions because of the severe packing constraints of the small lipid vesicles.

[5] Katz and Diamond (20) have reported that the water correction for liposome-water systems is complicated by the presence of "nonsolvent" water (i.e., an appreciable amount of water in the packed pellet not accessible to amphiphiles). Because the partition coefficients of both propranolol and timolol were significantly greater than 1, a water correction was not employed. Therefore, the total association of either propranolol or timolol as measured by radiolabeled compounds reflects the uncorrected (i.e., total) amount of labeled drug in the packed pellet and is expressed as moles of drug per mole of phospholipid. As there appears to be a greater association of both propranolol and timolol above than below the phase transition of DMPC (Fig. 4), while the water in the packed pellet above the phase transition is only one-half of what it is below the phase transition, this relatively small water correction would have little effect on the data obtained in the radiolabeled drug assays.

correction. The phospholipid content of the packed pellet was measured as total phosphorus, using a modification of the procedure of Chen *et al.* (21). More than 90% of the phospholipid was pelleted, implying that the vortexing procedure employed yielded mostly multilamellar lipid structures. In control experiments for the radioisotope measurements, the drugs were centrifuged in the absence of DMPC to show that the drugs do not form aggregated structures that are pelleted at the *g*-force employed in this study (22). However, sedimentation assays such as described here could be flawed if the drugs associated as aggregates with, but did not partition into, the membranes (10).

Calorimetric measurements. The thermotropic behavior of multilamellar DMPC in the presence and absence of drugs was observed in a high-sensitivity DSC (23). A scan rate of 0.5 K min^{-1} was used throughout, scanning from temperatures below the gel to liquid crystal phase transition to temperatures above the transition. The temperature, T_m, of maximal excess heat capacity was taken as a convenient measure of the transition temperature. The baseline for most of the calorimetric scans was readily established because there was no permanent change in heat capacity accompanying the transition. This allowed the transition enthalpy to be evaluated by planimeter integration of the scan. Cooling and rescanning of several of the samples showed that the transitions were fully reversible.

Neutron diffraction methods. DMPC and propranolol in a ratio 1.25:1 (mg/mg) in chloroform was prepared as described above (20 mM Tris, pH 7.5) with propranolol perdeuterated in the naphthalene moiety. Alternatively, DMPC was sonicated in a buffer solution (20 mM Tris, pH 7.5) of deuterated propranolol at 23° and used to make multilayers for diffraction. Control samples using fully protonated propranolol were prepared in an identical manner. These dispersions were centrifuged onto aluminum foil and glued to a glass slide (24). The glass slide was suspended over a saturated salt solution (66% relative humidity) and hydrated at different H_2O to D_2O ratios (25).

Lamellar neutron diffraction data were collected at the high-flux beam reactor using the Brookhaven low-angle diffractometer operating at 2.36 Å (26). The equipment design and detection system have been described previously (25). For these experiments the 2-dimensional position-sensitive counter had a spatial resolution of either 0.7 or 1.5 mm in the meridional (horizontal) and 1.5 mm in the equatorial (vertical) direction. The specimen geometry was similar to that previously described (25). Lamellar diffraction data (0.019 Å$^{-1} < s < 0.057$ Å^{-1}, where s is the reciprocal space coordinate) were collected at or above the phase transition temperature of DMPC at a specimen to detector distance of 80–85 cm via ω-scans ($0° \leq \omega \leq 6.0°$; $\Delta\omega = 0.1°$); one complete ω-scan required approximately 6 hr. Data reduction has been described in detail in a previous study (25). The lamellar reflections were phased by the swelling method for 100% H_2O data (27).

Materials. All reagents used were reagent-grade, and deionized water was glass-distilled prior to use. Unlabeled propranolol was purchased from Sigma Chemical Company (St. Louis, Mo.) and was used without further purification. [^{3}H]Propranolol (19.0 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.), and ^3H_2O (0.1 Ci/mmol) was obtained from ICN (Irvine, Calif.). Unlabeled timolol and [^{3}H]timolol (34.7 Ci/mmole) were gifts from Merck Sharp & Dohme (Rahway, N. J.). DMPC was purchased from Calbiochem-Behring Company (San Diego, Calif.). Deuterated propranolol was obtained from Merck Sharp & Dohme (Canada).

RESULTS

Calorimetric studies with a model membrane system (DMPC). Both propranolol and timolol, when mixed with multilamellar DMPC as described above, lowered the transition temperature of the lipid. As illustrated in Fig. 1, this lowering was proportional to drug concentrations up to approximately 3 mM propranolol and 30 mM timolol. At high drug concentrations the transition temperature lowerings were less than expected from the least-square lines in Fig. 1, which could be described by the

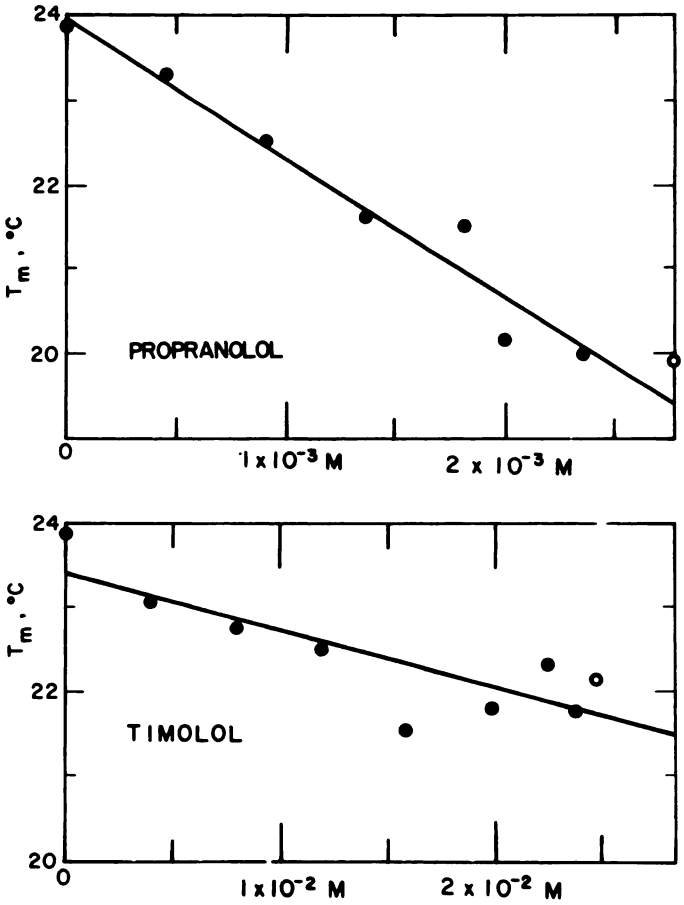

FIG. 1. *Plots of T_m of DMPC as a function of the total drug concentration for propranolol and timolol*

DMPC concentration was 1 mg/ml in 0.01 M Tris buffer at pH 7.0 or pH 7.5. The point represented in each graph by ○ was obtained with a suspension prepared by vortexing the dry lipid at 45–50° in a solution of the drug.

equations

$$T_m = (23.96 \pm 0.10) - (1.64 \pm 0.08)C_2 \quad (1)$$

$$T_m = (23.40 \pm 0.11) - (0.07 \pm 0.01)C_2 \quad (2)$$

for propranolol and timolol, respectively. In these equations C_2 is the total millimolar concentration of the drug.

Typical DSC curves showing the variation of excess heat capacity with temperature are given in Fig. 2. The noise level in the original traces was approximately 0.05 kcal K^{-1} mole^{-1} peak to peak. The shapes of these curves indicate that these are not simple systems in which the solute forms an ideal solution in the liquid (liquid crystal) phase and is insoluble in the solid (gel) phase (28). If such were the case, the two curves in Fig. 2, which were selected to show equal lowering of transition temperature, should have the same asymmetrical shape, with marked broadening on the low temperature side (29). Furthermore, although it is probable that both drugs have some solubility in the gel phase, neither curve can be fitted on the assumption that ideal solutions are formed in both the liquid crystal and gel phases (29). Thus, the curve for propranolol has the shape expected for a solute having higher solubility in the gel phase than

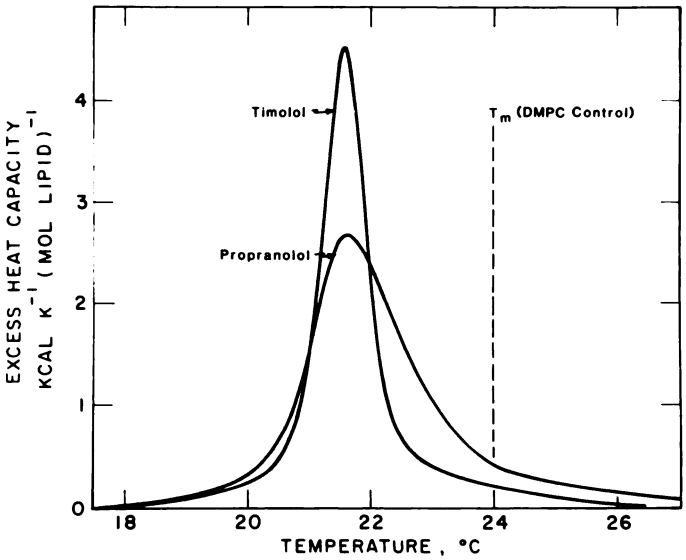

FIG. 2. *DSC transition curves for propranolol at a total concentration of 1.9 mM and a DMPC concentration of 1.00 mg/ml, and for timolol at a total concentration of 16 mM and a DMPC concentration of 1.26 mg/ml*

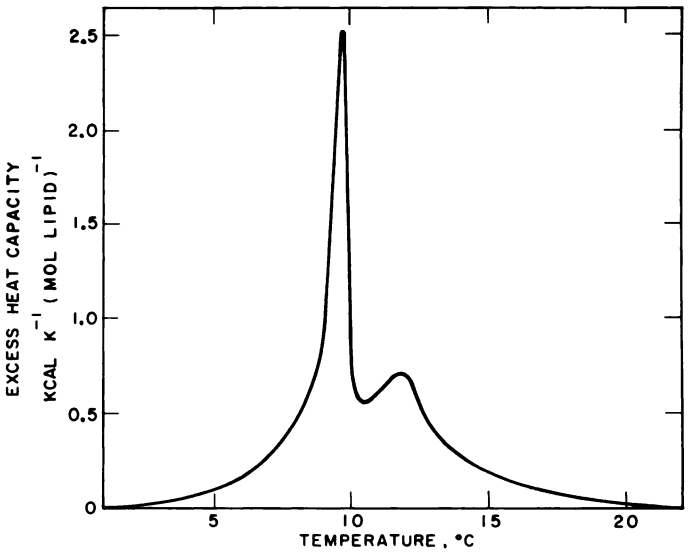

FIG. 3. *DSC curve observed with 29 mM propranolol and a DMPC concentration of 1.0 mg/ml*

in the liquid crystal phase; if that were actually the case, ideal solution theory would predict an increase in the transition temperature. Similarly, the nearly symmetrical curve for timolol with a significant lowering of the transition temperature cannot be accounted for on the basis of ideal solution theory.

Although these clear indications of nonideality in these drug-lipid systems show that the calorimetric studies cannot yield quantitative estimates of the partitioning of the drugs between the aqueous and lipid phases, the large ratio, amounting to 25, of the slopes given in Eqs. 1 and 2 indicates that propranolol is probably much more soluble in DMPC than is timolol. It may be assumed that, whatever their actual values, the partition coefficients remain essentially constant over the concentration ranges where the transition temperatures vary linearly with drug concentration.

The enthalpies of transition per mole of DMPC were constant within experimental error over the range of concentrations shown in Fig. 1. The mean value for the propranolol mixtures was 5.83 ± 0.13 kcal mole^{-1}, and for the timolol mixtures it was 5.65 ± 0.05 kcal mole^{-1}. This constancy of enthalpies suggests that the deviations from ideality in the gel and liquid crystal phases are similar, since there appears to be no significant enthalpy change accompanying the transfer of drug from gel to liquid crystal phase during the phase transition. For propranolol concentrations higher than those in Fig. 1, the enthalpies decreased, reaching a minimum at 10 mM (T_m = 16.3°, ΔH = 3.32 kcal mole^{-1}), and then increased to 4.74 kcal mole^{-1} at 29 mM, the highest concentration employed. This latter mixture showed a transition curve with a major peak at 9.3° and a minor one at 11.8° (Fig. 3). The peak at 9.3° was quite sharp, with a van't Hoff enthalpy of 750 kcal mole^{-1}. This peak may correspond to some sort of packing complex between the drug and the lipid. Concentrations of timolol higher than those in Fig. 1 were not investigated.

Radioisotope measurements of drug association with model membrane systems. These experiments were carried out at temperatures and concentrations of lipids and drugs similar to those utilized in some of the calorimetric studies described above. The results of an experiment in which either propranolol or timolol was incubated with dispersions of DMPC above and below the phase transition temperature are summarized in Fig. 4. (In this and subsequent figures, drug associated with lipid was quantified by the molar association ratio for radioisotope studies and expressed as moles of drug per mole of phospholipid.) When concentrations of propranolol (0.34 mM) and timolol (3.2 mM) were chosen to yield similar total association of drugs with the lipid, molar associations for both drugs were greater above than below 23.8°, the phase transition temperature of DMPC (Fig. 4). At similar drug concentrations (0.3–0.4 mM), approximately

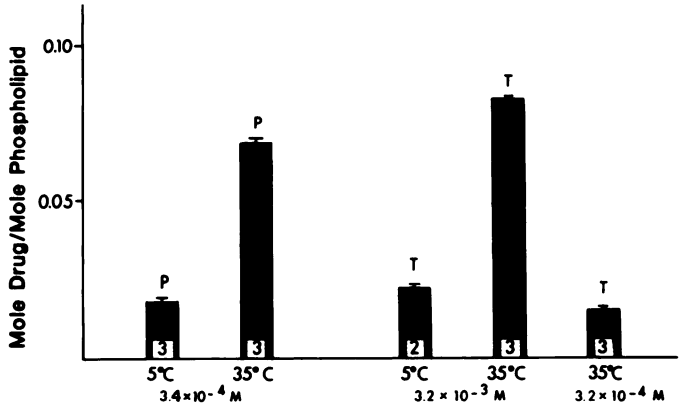

FIG. 4. *Association of propranolol and timolol above and below the phase transition temperature (23.8°) of DMPC utilizing radioisotope measurements*

Comparison of propranolol (0.34 mM) and timolol (3.2 mM) shows differences of association to DMPC at 5° and 35°. In the *right-hand bar*, timolol (0.32 mM) was added at a concentration similar to that of propranolol at 35° for comparison. The *numbers* at *bottoms* of bars refer to the numbers of experiments for a single determination.

5 times as much propranolol as timolol was associated with the phospholipid (Fig. 4). Results obtained at 5° using dipalmitoyl lecithin, with a phase transition at 41°, demonstrated even smaller association ratios for both propranolol and timolol (data not shown). This might be expected, since the temperature at which the experiment was carried out (5°) is further below the phase transition temperature of dipalmitoyl lecithin than DMPC. When these drugs were incubated with DMPC at the phase transition temperature of pure DMPC (23°), the molar association of propranolol remained greater than that of timolol, as shown in Fig. 5.

Neutron diffraction studies with a model membrane system. Neutron diffraction with propranolol deuterated in the naphthalene moiety was employed to define the location of this drug molecule in the DMPC lipid bilayer (30). The unit cell dimensions for DMPC bilayers with and without propranolol differed by less than 1 Å, suggesting that propranolol did not cause significant swelling of the lipid bilayer. Significant differences in the lamellar intensities were observed for DMPC lipid bilayers with protonated versus deuterated propranolol. This study was carried out at 23° and repeated at 30° with similar results under conditions in which the DMPC bilayer was in the liquid crystal state. This was confirmed by X-ray diffraction since there was no indication of a sharp 4.2-Å reflection (gel packing). The lamellar meridional neutron diffraction pattern contained only three *prominent* orders which could be used in this analysis, probably due to the DMPC's being above its phase transition. Even though the resolution is quite low, it is sufficient for a determination of the average position of both the phospholipid headgroups and naphthalene moiety of propranolol within the corresponding unit cell profile structures. The fine structure details of the hydrocarbon core of the DMPC lipid bilayer are not evident at this resolution (Fig. 6). The neutron scattering difference profile function for propranolol deuterated (naphthalene moiety) versus protonated, calculated from these differences in lamellar intensities, demonstrated average peak densities at ±9 Å about the center of the hydrocarbon core of the DMPC lipid bilayer (Fig. 6). This value corresponds to the separation of the central maxima within the difference profile for deuterated versus protonated propranolol given in Fig. 6 (real-space approach). It has been shown that a reciprocal space approach in which the difference structure factors are model-refined can provide a more accurate determination of these peak locations (31). This procedure showed that the peak densities attributable to the deuterated naphthalene moiety of propranolol were located 11 ± 1 Å from the center of the

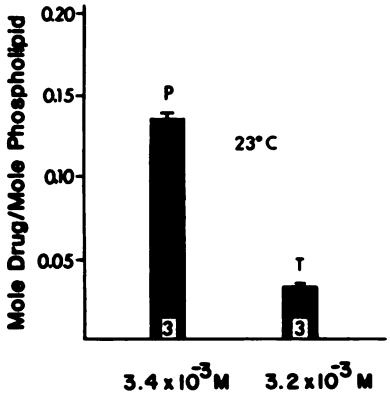

FIG. 5. *Association of 3.4 mM propranolol and 3.2 mM timolol with DMPC utilizing radioisotope measures*
Conditions as in Fig. 4 except that the temperature was 23°.

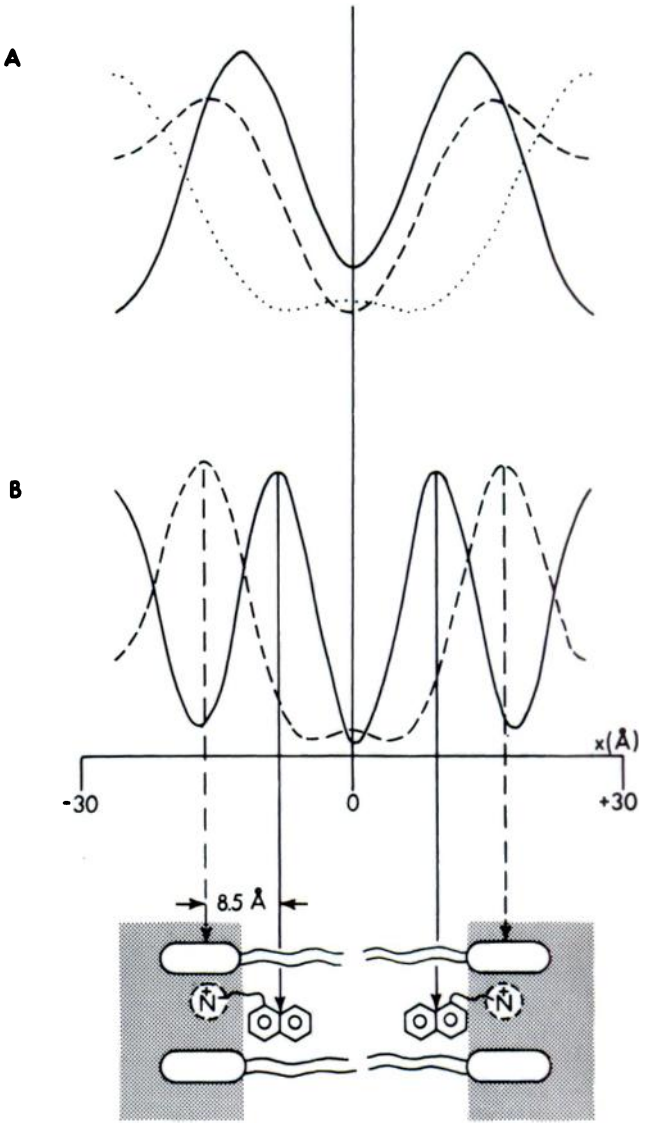

FIG. 6. *Resolution neutron-scattering profiles for the propranolol-DMPC system at 23°*

A. Lower resolution neutron-scattering profiles for the propranolol-DMPC system at 23°. ---, The scattering profile of DMPC with protonated propranolol in 100% H_2O which provides the positions of the headgroups of the DMPC lipid bilayer. ···, The water profile structure obtained by H_2O/D_2O exchange. ——, The neutron-scattering difference profile for DMPC lipid bilayers with propranolol deuterated versus protonated in the naphthalene moiety.

B. The same profiles as above at slightly higher resolution (18 Å) (the water profile is omitted for clarity). The average location of the naphthalene moiety of propranolol (peak densities 11 ± 1 Å about the center of the DMPC hydrocarbon core) is mapped directly along the profile axis of the DMPC lipid bilayer. The increase in density at the edges of the unit cell (± $D/2$) within this difference profile is attributed to Fourier truncation artifacts, since the lamellar intensity data set for deuterated propranolol had a very weak third-order reflection relative to the corresponding lamellar intensity data set for protonated propranolol.

hydrocarbon core of the DMPC lipid bilayer. In separate experiments carried out on the same samples, H_2O/D_2O exchange was used to obtain a difference profile structure, which corresponds to the water profile structure. This water profile structure was used to define the hydrocarbon core of the DMPC lipid bilayer, from which water was excluded. When the average peak densities for the naphthalene moiety of propranolol were mapped along the profile axis of the DMPC lipid bilayer, the naphthalene moiety of the propranolol molecule was found to be within the hydrocarbon core region of the DMPC lipid bilayer (Fig. 6). This location of the naphthalene moiety would position the charged amine moiety of propranolol within the water layers hydrating the phospholipid head groups of the DMPC lipid bilayer (see Discussion). This result, which is consistent with the calorimetric and radioisotope measurements, supports the view that the lowering of the phase transition temperature of DMPC was due to direct solvation of propranolol into the DMPC lipid bilayer. Corresponding neutron diffraction measurements for timolol have not yet been made, owing to the difficulty of deuterating this compound.

Radioisotope measurements of the drug association with the SR membrane. The molar association ratio, expressed as moles of drug per mole of total SR phospholipids (assuming an average molecular weight of 760 for SR phospolipids), was larger with propranolol than with timolol when the closed membrane vesicles of light SR were incubated with equal concentrations of these drugs (Fig. 7). Experiments with deproteinated, highly purified SR lipids indicated that removal of protein significantly ($p < 0.001$) enhanced the association of the drugs, raising the molar association ratio to values similar to those observed with DMPC above its phase transition temperature. This is not surprising since, according to X-ray diffraction data (24, 32), the purified SR lipids remain in the liquid crystal state at temperatures as low as −5°.

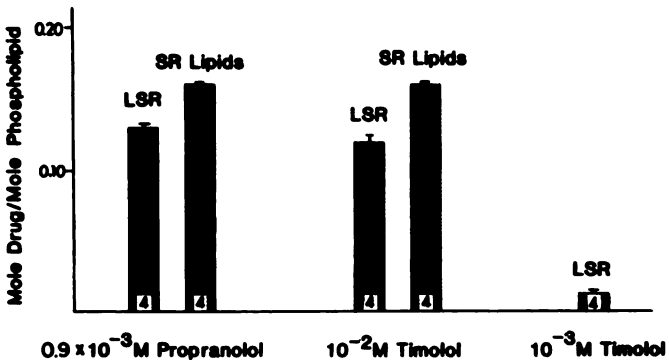

FIG. 7. *Association of propranolol and timolol with the highly purified light fraction of SR membranes (LSR) and highly purified SR lipids at 10°*

Concentrations were as follows: propranolol, 0.9 mM; timolol, 10 mM; SR membranes, 1.25 mg of proteins per milliliter; and SR lipids, 0.88 mg/ml. The *right-hand bar* shows the effects of 1.0 mM timolol incubated with SR for comparison with the same concentration of propranolol. The amount of phospholipid in the SR membrane and SR lipid experiments was equivalent.

The concentration dependence of the molar association ratio of these drugs for light SR membranes is shown in Fig. 8, which also shows the inhibitory effects of these drugs on calcium uptake previously reported by Messineo and Katz (4). In this study, carried out at 20° to facilitate comparison with the concentration dependence of the inhibition of initial calcium uptake velocity by these drugs (4), concentrations of propranolol (Fig. 8A) significantly lower than the concentrations of timolol (Fig. 8B) were found to be associated with the light SR membrane.

Because of the solubility properties of both propranolol and timolol in aqueous media, only a portion of the

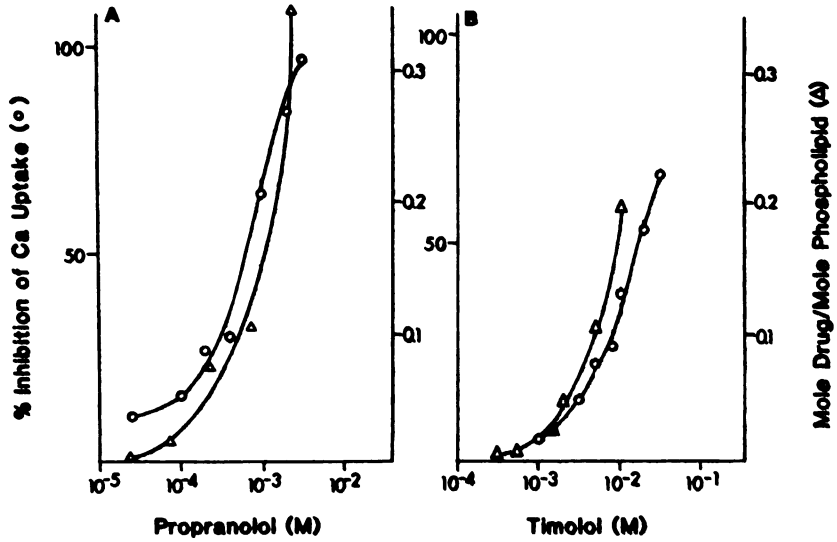

FIG. 8. *Concentration dependence of the association of propranolol (A) and timolol (B) with isolated SR membranes at 20° via radioisotope measurements (△)*

Because of the solubility of these drugs in an aqueous medium the association curves are necessarily incomplete. Determinations of the concentration dependence of the effects of propranolol and timolol on initial calcium uptake velocity (○), expressed as a percentage of control velocity [data from Messineo and Katz (4)], have been superimposed on the association curves.

concentration dependence of the association of these drugs with the SR membrane could be explored. Double-reciprocal plots of the concentration dependence of the molar association of propranolol and timolol with the SR membrane over this limited concentration range (Fig. 9) provided estimates of the concentrations at which half-maximal association with the SR membrane occurs, being 0.87 mM for propranolol as compared with 36 mM for timolol. This value for propranolol was similar to the propranolol concentration that caused 50% inhibition of initial calcium uptake velocity by SR (I_{50} = 0.65 mM). The I_{50} value for propranolol was determined over a concentration range of 0–100% inhibition of calcium uptake velocity by SR (4). However, the concentration for half-maximal association of timolol with the SR membrane (36 mM) was more than 3 times greater than the concentration required for 50% inhibition of calcium transport (I_{50} = 11 mM). The I_{50} value for timolol was determined over a concentration range of 0–70% inhibition of calcium uptake velocity by SR, since the relatively high timolol concentrations needed for complete inhibition could not be attained because of the solubility properties of timolol in aqueous media (4). In addition, the I_{50} values for the inhibitory effects of these drugs obtained by Messineo and Katz (4), which differ by a factor of approximately 17, are similar to the ratio of the slopes (equal to 23) for propranolol versus timolol given in Fig. 1, obtained by differential scanning calorimetry utilizing the model membrane DMPC (see above), but are significantly less than the ratio of 41 obtained by radioisotope measurements of the association of these drugs with the SR membrane.

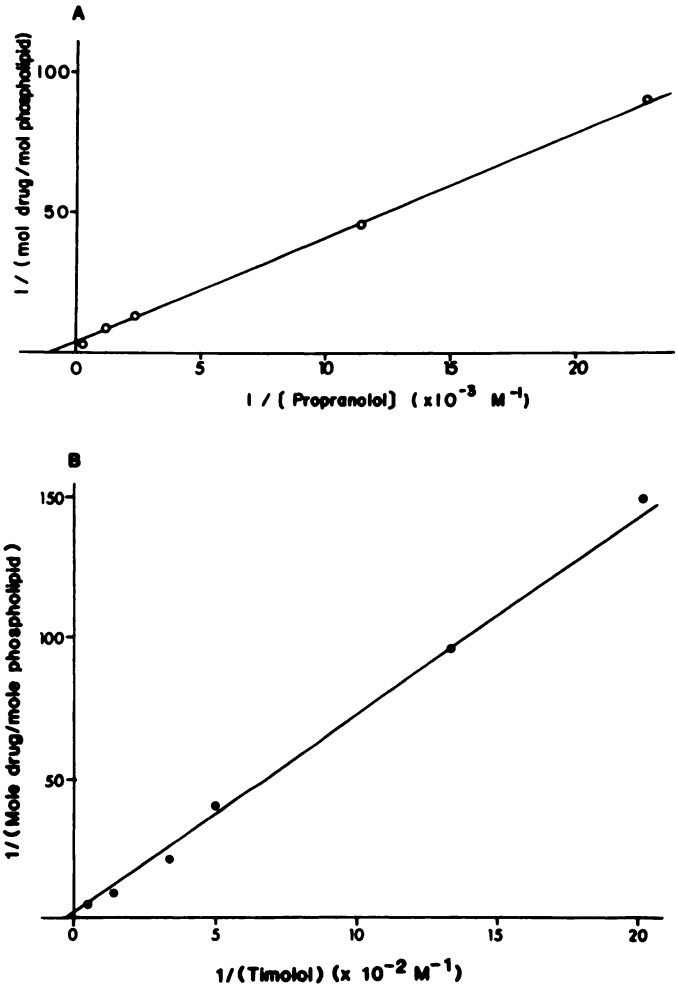

FIG. 9. *Double-reciprocal plots of the drug concentration dependence of the total molar association ratio for propranolol (A) and timolol (B) obtained from Fig. 8*

The K_a values were obtained by a least-squares fit to the data shown.

DISCUSSION

Interaction of propranolol and timolol with model membranes. The structural basis for the nonspecific interactions of propranolol and timolol with the SR membrane was initially approached by utilizing high concentrations of these *beta*-adrenergic receptor-blocking drugs and a well-defined model membrane (DMPC). The association of radioisotope-labeled propranolol and timolol with DMPC was greater for both drugs in the liquid crystal state than in the gel state (Fig. 4), indicating that the association depends at least in part on the physical state of the fatty acyl chains. Measurements at 23° indicate that influences other than the lipid phase transition, such as different temperature coefficients of partition coefficients, cannot contribute significantly to the differences shown in Fig. 4. These radioisotope measurements define only the total association of the drugs with the model membrane system and provide no information concerning the precise localization of the drugs within the lipid bilayer (10). Such measurements are subject to some uncertainty because of the difficulty in correcting for drug trapped in the interstitial water spaces of the packed pellet (ref. 19; footnote 4).

In comparing the results of the radioisotope experiments with those obtained by scanning calorimetry, it must be remembered that, as explained earlier, it is impossible to draw quantitative conclusions from the calorimetric experiments. Nevertheless, the calorimetric data are consistent with the radioisotope results in indicating that both propranolol and timolol partition into the lipid bilayer, thereby lowering its phase transition temperature, and that the interaction of timolol with DMPC is less pronounced than that of propranolol.

The radioisotope measurements indicate that the concentrations of propranolol in the lipid, both below and above the phase transition temperature, are 5–7 times those of timolol, a ratio that is much smaller than the ratio of the calorimetrically determined slopes equal to 23 given in Eqs. 1 and 2. The ratio of the slopes of the curves in Fig. 1 reflects the ratio of the relative solubilities of these drugs in the DMPC model membrane system. It thus appears that at a given concentration in the lipid, timolol is less effective than propranolol in lowering the transition temperature. This difference cannot be attributed to a relatively greater partitioning of timolol into the gel phase as compared with the liquid crystal phase since the radioisotope data indicate similar ratios for the concentrations in the two phases.

It appears that propranolol and timolol are positioned differently within the bilayer with the result that timolol has less influence on the phase transition. This could

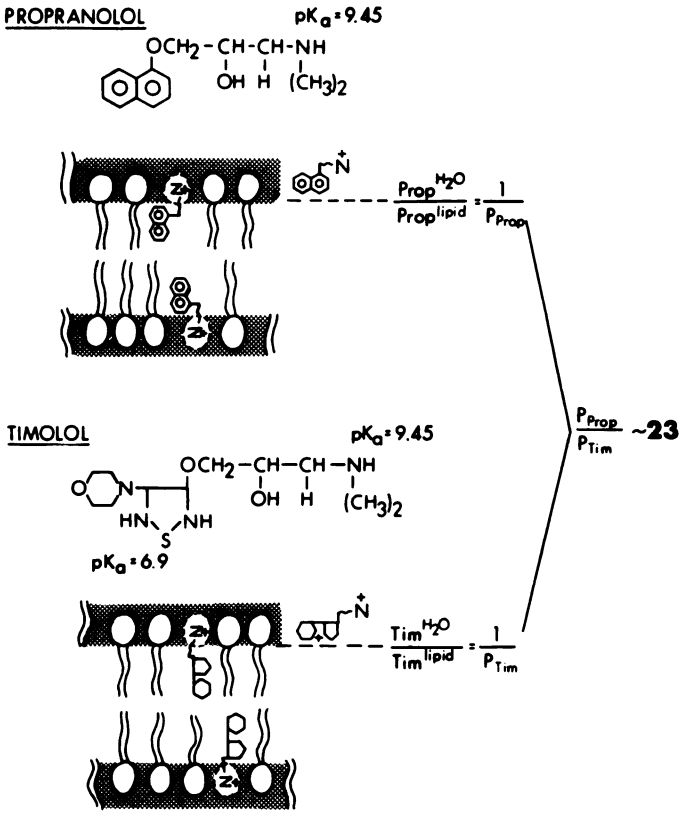

FIG. 10. *Schematic representation of the localization of propranolol and timolol in the model membrane system depicted as a partitioning between the lipid and aqueous phases of this system*

The naphthalene ring of propranolol anchors the charged amine side chain as shown. The partially charged morphine ring of timolol, in contrast to the aromatic naphthalene ring of propranolol, may explain the dramatic partitioning (aqueous/hydrocarbon core) difference for these two *beta*-receptor-blocking drugs, which is approximately a factor of 23; however, timolol would appear to have another site of interaction in addition to the less complex partitioning of propranolol.

occur if more timolol was localized in the head group region than is propranolol. A possible explanation of such a difference is provided by the charge characteristics of the two drugs (13, 33), since the ionic strength and pH were the same for the radioisotope, calorimetric, and neutron diffraction experiments. Propranolol has been reported to have a pK_a of 9.45 (13), which is identical with that for the amino group in timolol,[6] and the pK_a for the morphine ring in timolol is 6.9. At the pH of the experiments with model membranes (pH 7.5), therefore, the additional charge on the timolol molecule could reduce its average penetration into the nonpolar portion of the bilayer (Fig. 10). A similar picture has been inferred from pH gradient studies, in which propranolol but not timolol was found to alter the transmembrane pH of liposomes (34). This earlier study also concluded that propranolol in the charged form was bound to the membrane and in the uncharged form penetrated into the membrane (34). Neutron diffraction utilizing deuterated timolol will be needed to define further timolol-membrane interaction.

It is not possible to exclude an additional explanation, that differences in the interactions of propranolol and timolol with membranes are due to different monomer-oligomer equilibria in the two cases. The neutron diffraction study, which showed an intercalation of propranolol into the lipid bilayer, suggests that this is not the case for propranolol. The greater charge density of timolol makes it unlikely that timolol would form aggregated structures even at the relatively high concentrations used.

Interaction of propranolol with model membranes studied by neutron diffraction. Analysis of the neutron diffraction data obtained with protonated and deuterated propranolol incorporated into DMPC bilayers indicates that the napthhalene moiety of propranolol is localized within the hydrocarbon core of the bilayer (Fig. 6). This orientation of the naphthalene moiety implies that the side chain in the propranolol molecule should be all-*trans* in order to position the charged amino group within the aqueous region surrounding the phospholipid head groups, as shown in Fig. 11. Thus, the maximal extension for an all-*trans* side chain is calculated to be 6 Å, which positions the charged amino group very near to that of the phosphate moiety of the lipid head group (Fig. 11).[7] This observation supports the interpretations of the radioisotope and calorimetric studies that propranolol interacts mainly with the nonpolar part of the lipid bilayer. The possibility that some part of the observed effect of propranolol on the lipid phase transition is caused by electrostatic contributions arising in the headgroup region cannot be excluded by any of our results. The localization proposed for propranolol within the bilayer (Fig. 6) is similar to that suggested for tetracaine on the basis of nuclear magnetic resonance studies (33).

Interaction of propranolol and timolol with the SR membrane. The total association of both propranolol and timolol with the SR membrane was measured only by the radioisotope method. It has been shown by X-ray diffraction (24, 25, 32) that the heterogeneous fatty acyl chain mixture comprising the lipid bilayer of the SR membrane does not have a melting point, as evidenced by the persistence of a broad 4.6-Å reflection at temperatures as low as −5°. These studies, therefore, indicate that the lipid chains of the SR membrane remain in a melted state, precluding the use of calorimetric methods.

The radioisotope measurements summarized in Fig. 7 show that both drugs have a lesser association with intact SR membranes than with deproteinated, highly purified SR lipids, where the amount of phospholipid in each experiment was equivalent. This difference can be explained if each molecule of the calcium pump protein rendered approximately 32 phospholipid molecules inaccessible for interaction with these drugs. This amount

[6] C. S. Sweet (Merck Sharp & Dohme, Rahway, N. J.), personal communication.

[7] From the detailed analysis of Buldt *et al.* (31), it can be shown that the phosphate moiety of the phospholipid headgroup occurs at the center of the maxima which correspond to the phospholipid headgroups within the neutron-scattering profile. Our analysis indicates that the central (phospholipid headgroup) maxima occur at ±17 Å about the center of the hydrocarbon core of the DMPC lipid bilayer. The reciprocal space approach indicates that the center of mass of the naphthalene moiety is 11 ± 1 Å about the hydrocarbon core center. Thus, the calculated all-*trans* side chain extension of 6 Å for propranolol would position the charged amino group directly in line with the phosphate moiety of the DMPC headgroups.

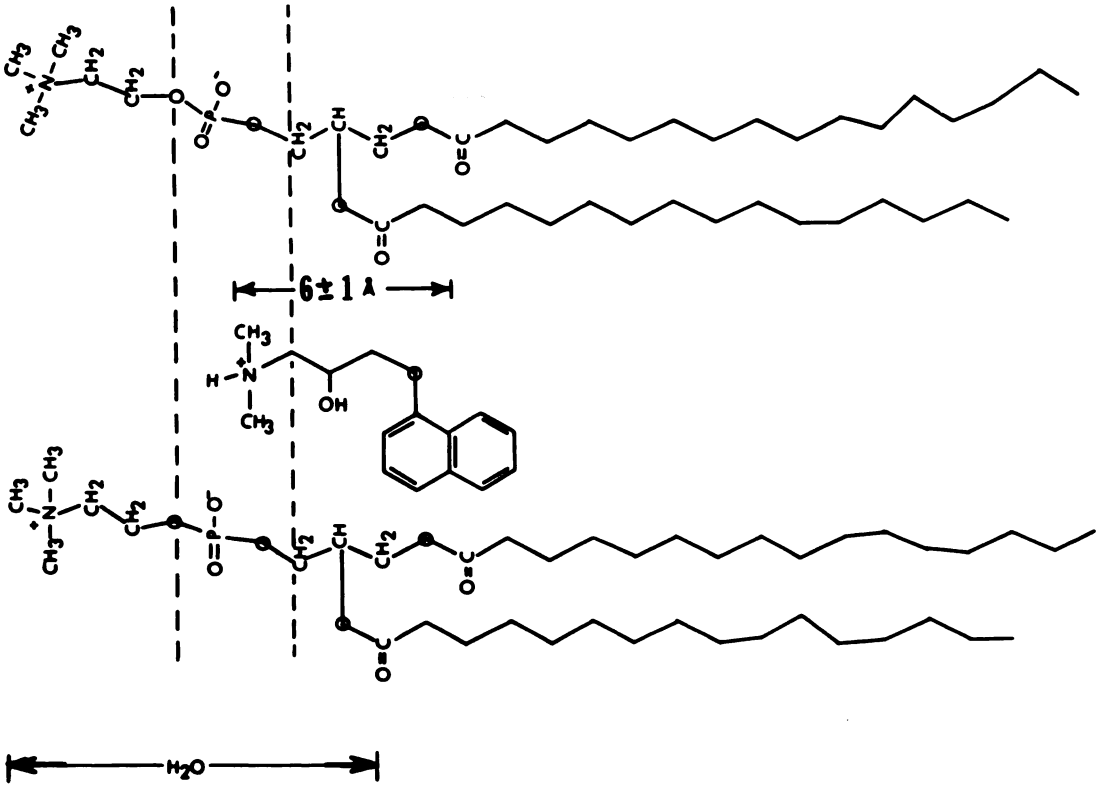

FIG. 11. *Localization of the propranolol molecule within the DMPC lipid bilayer as determined by neutron diffraction*
The distance between the center of the naphthalene moiety and the charged amine group is 6–8 Å.

of lipid has been shown to be the minimal requirement for optimal ATPase activity of the pump protein in SR (35).

In accord with the studies of DMPC vesicles, the radioisotope measurements showed that propranolol associates with the SR membrane to a greater extent than does a similar concentration of timolol (Fig. 7). In addition, the associations of these drugs with both intact SR and its purified lipid component were similar to those found with DMPC *above* its phase transition. For example, at 0.34 mM propranolol, a molar ratio of 0.075 (Fig. 4) is obtained with DMPC at 35°, whereas at the same drug concentration, with intact SR, a molar ratio of 0.06 (Fig. 8) is found; by inference from a comparison of Figs. 7 and 8, a molar ratio of approximately 0.07–0.08 should be found for SR lipids at approximately 0.4 mM propranolol. Since propranolol interaction with the DPPC model membrane system (multilamellar structures) is quantitatively similar to that in intact SR vesicles (unilamellar), to a first approximation, the localization of the naphthalene moiety in the SR membrane is inferred to be the same as its localization in the model membrane system.

The differences in the potencies of propranolol and timolol to inhibit initial calcium uptake velocity by the SR membrane (4) resemble their different association with these membranes and the association of these drugs with the model membrane system. Messineo and Katz (4) showed that calcium uptake velocity was inhibited 50% by 0.65 mM propranolol and 11 mM timolol,[8] whereas double reciprocal plots of the concentration dependence of the *total association* of these drugs with the SR membrane (Fig. 9) indicate that the half-maximal concentration for association of propranolol is 0.87 mM while that for timolol is 36 mM. The similarities between the concentrations for the inhibitory effects of propranolol on calcium transport (I_{50} value) and its association with the SR membrane in light of the structural studies with the model membrane system provide further evidence that calcium uptake inhibition is related to the incorporation of the drug within the SR membrane. Because propranolol is much more lipid-soluble than timolol (as shown by both radioisotope and calorimetric data) inhibition of calcium uptake by propranolol may be directly related to solvation of the drug in the lipid bilayer of the SR membrane (Fig. 12). The finding that the I_{50} value for inhibition of calcium uptake by timolol is substantially lower than the concentration for half-maximal association measured by the radioisotope method, whereas these values are similar in the case of propranolol, is consistent with other evidence that the interaction of timolol with the SR membrane, like that with the model membrane system, is more complex than propranolol-membrane interactions and may involve more than one site (see Fig. 10). The present data suggest that this second site of interaction of timolol with the SR membrane is without effect on calcium transport.

[8] Using a spectrophotometric approach (24) for measuring calcium uptake by SR, we have determined I_{50} values for propranolol and timolol equal to 0.79 and 18.6 μM, respectively, similar to the values obtained by Messineo and Katz (4) using a radioisotope method. These I_{50} values differ by a ratio of 24, similar to the ratio of the DSC slopes in Fig. 1.

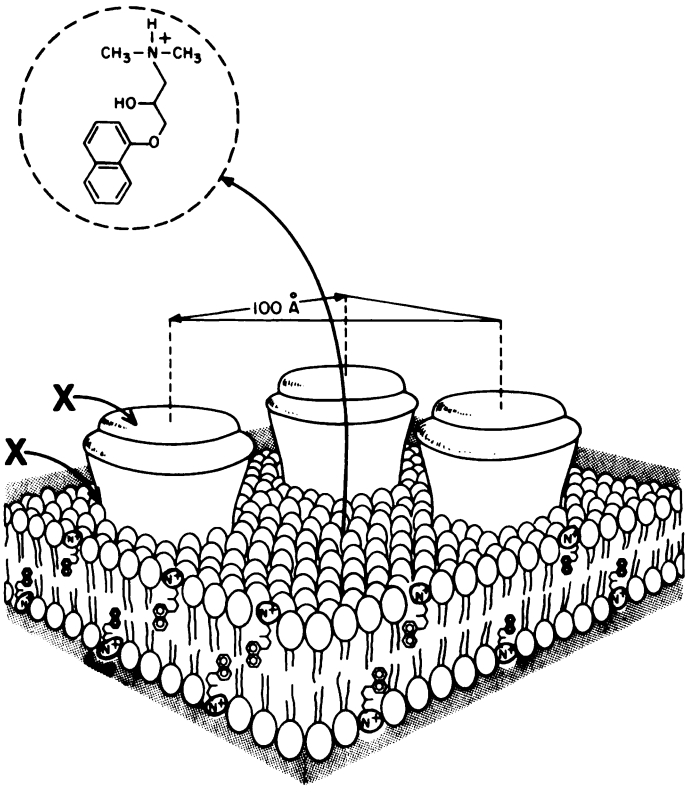

FIG. 12. *Schematic representation depicting the interaction of propranolol with the SR membrane*

The predominant interaction of propranolol with the SR membrane is shown as a solvation of the drug within the hydrocarbon core of the SR membrane bilayer, and thus propranolol may perturb the functional mechanism of the calcium pump protein indirectly via the *bulk lipid matrix* of this membrane. Other sites of drug interaction (×), namely, with the lipid annulus of the calcium pump protein and directly with the calcium pump protein itself, apparently do not occur (at least for propranolol). Further details of the SR membrane structure are given elsewhere (24, 36).

The differences between the interactions of propranolol and timolol with the simple membranes examined in this study have several implications regarding their effects in more complex systems. For example, the exchange of a drug across the capillary between the plasma and the interstitial fluid may be influenced by its ability to incorporate into the capillary membrane. The relative lipid solubilities of propranolol and timolol observed in this study, therefore, are consistent with a report that timolol is less able to cross the blood-brain barrier than is propranolol (34). This interpretation is supported by the extensive tissue binding of propranolol (37) and the data of Tocco *et al.* (38), who found that propranolol concentrations in the brain were at least one order of magnitude higher than those of timolol. Although the present studies employed drug concentrations well above those needed to block *beta*-adrenergic receptors, the data that we have obtained provide a structural basis for the view that at least some of the nonspecific effects of these drugs may be related to membrane effects that are distinct from their *beta*-adrenergic-blocking potencies. These findings also suggest that timolol, which is less lipid-soluble, is a more specific *beta*-adrenergic receptor blocker than is propranolol.

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