Interaction of the NMDA Receptor Noncompetitive Antagonist MK-801 with Model and Native Membranes

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ABSTRACT MK-801, a noncompetitive antagonist of the NMDA (*N*-methyl-D-aspartate) receptor, has protective effects against excitotoxicity and ethanol withdrawal seizures. We have determined membrane/buffer partition coefficients ($K_{p[mem]}$) of MK-801 and its rates of association with and dissociation from membranes. $K_{p[mem]}$ (±SD) = 1137 (±320) in DOPC membranes and 485 (±99) in synaptoneurosomal (SNM) lipid membranes from rat cerebral cortex (unilamellar vesicles). In multilamellar vesicles, $K_{p[mem]}$ was higher: 3374 (±253) in DOPC and 6879 (±947) in SNM. In cholesterol/DOPC membranes, $K_{p[mem]}$ decreased as the cholesterol content increased. MK-801 associated with and dissociated from membranes rapidly. Addition of ethanol to SNM did not affect $K_{p[mem]}$. MK-801 decreased the cooperative unit size of DMPC membranes. The decrease was smaller than that caused by 1,4-dihydropyridine drugs, indicating a weaker interaction with the hydrocarbon core. Small angle x-ray diffraction, with multilayer autocorrelation difference function modeling, indicated that MK-801 in a cholesterol/DOPC membrane (mole ratio = 0.6) causes a perturbation at ~16.0 Å from the bilayer center. In bilayers of cholesterol/DOPC = 0.15 (mole ratio) or pure DOPC, the perturbation caused by MK-801 was more complex. The physical chemical interactions of MK-801 with membranes in vitro are consistent with a fast onset and short duration of action in vivo.

INTRODUCTION

MK-801 (dizocilpine; (5R, 10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine) is a noncompetitive antagonist of the NMDA (*N*-methyl-D-aspartate) receptor (Wong et al., 1986), a subtype of glutamate receptor that is associated with a cation channel. The chemical structure of MK-801 is shown in Fig. 1. The (+)-isomer (shown) is more active than the (-)-isomer. The NMDA receptor-ion channel complex has several binding sites, including sites for 1) agonists (i.e., glutamate, NMDA), 2) glycine, 3) phencyclidine (PCP), 4) Mg^{2+}, 5) Zn^{2+}, and 6) polyamines (Kozikowski et al., 1989). MK-801 binds to the PCP-binding site, which is believed to lie within the ion channel, deep enough to be at the level of the hydrophobic core of the membrane bilayer (Sakurada et al., 1993). Thus, efficiency of interaction of MK-801 with its receptor could be affected by the structure and composition of the membrane, especially by the concentration and distribution of membrane cholesterol. MK-801 is known to penetrate the central nervous system (Wong et al., 1986). It is thought to approach its specific binding site through the open cation channel, because the presence of an agonist is required for MK-801 binding (Foster and Wong, 1987; Reynolds and Miller, 1988). The NMDA receptor complex has been implicated in excitotoxicity caused by ischemia, hypoglycemia, and other causes, and antagonists of this receptor, including MK-801, have been shown to have neuroprotective effects (Gill et al., 1988; Schwartz and Meldrum, 1986, Tasker et al., 1992; el-Asrar et al., 1992). MK-801 appears to protect against other types of neurotoxicity as well, such as that induced in dopaminergic systems by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Tabatabaei et al., 1992; Zuddas et al., 1992). It has anticonvulsant and sympathomimetic effects and may also have anxiolytic properties (Clineschmidt et al., 1982a–c; Xie and Commisaris, 1992; Rupniak et al., 1993). The action of agonists at the NMDA receptor is inhibited by acute ethanol administration (Hoffman et al., 1990; Lovinger et al., 1990). This receptor is up-regulated after chronic ethanol consumption and is involved in the generation of withdrawal seizures (Grant et al., 1990). MK-801 can prevent the development of tolerance to ethanol, cocaine, and morphine (Khanna et al., 1992; Wu et al., 1993; De Montis et al., 1992; Trujillo and Akil, 1991) and can reduce the severity of ethanol withdrawal seizures (Grant et al., 1990). Because MK-801 or similar agents could eventually be useful in prevention or treatment of the conditions discussed here, knowledge of its interaction with the membrane would be useful for the design of new drugs with optimal membrane/receptor properties. We have investigated the interaction of MK-801 with native and model membranes, especially with regard to the effects of membrane cholesterol content.

MATERIALS AND METHODS

Materials

Phospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, assay reagents, salts, solvents, and buffers were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]MK-801 and [^{14}C]DOPC were purchased from NEN Research Products (Boston, MA). Whatman GF/C glass fiber filters (FP-205) were obtained from Brandel (Biomedical Research and Development Laboratories, Gaithersburg, MD).

Received for publication 3 June 1994 and in final form 24 August 1994.

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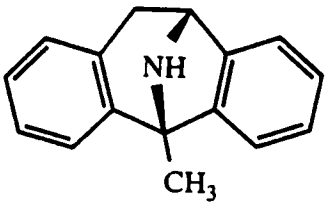


MK-801

FIGURE 1 The chemical structure of MK-801. MK-801 is a bulky, rigid molecule with a single nitrogen atom that can be protonated. The more active, (+)-isomer is shown. The unlabeled form used in this work was the hydrogen maleate. The radiolabeled form was probably the acetate.

Preparation of SNM and extraction of SNM lipids

SNM were prepared from rat cerebral cortex by a modification of the original method (Hollingsworth et al., 1985) as previously reported (Moring et al., 1991). Male Sprague-Dawley rats were obtained from Zivic-Miller (Zelienople, PA). Protein concentration was assayed by the method of Lowry et al. (1951); phospholipid concentration was estimated from the inorganic phosphate content as determined by the method of Chen et al. (1956).

Lipid extraction was performed by the method of Sun and Lin (1989). In an acid-washed glass centrifuge tube, 4 vol of 2/1 (v/v) chloroform/methanol was added to the SNM preparation. The mixture was vortexed and centrifuged for 2 min at low speed to separate the phases (setting 5, IEC Clinical Centrifuge, International Equipment Co., Needham Heights, MA). The organic phase was removed to another tube. Two vol (original preparation) of 2/1 chloroform/methanol, 0.4% HCl, was added to the aqueous phase, and the mixture was vortexed and centrifuged as before. The organic phase was neutralized with one drop of 4N NH_4OH and vortexed. The organic phases were combined, evaporated to dryness under a stream of nitrogen, and immediately reconstituted to 2 2/3 times the volume of the original SNM preparation using 2/1 chloroform/methanol. Phospholipid concentration was estimated from inorganic phosphate content determined by the method of Chen et al. (1956). Cholesterol content of the lipid extracts was assayed enzymatically by the method of Heider and Boyett (Heider and Boyett, 1978) as modified by Chester (Chester et al., 1986).

Preparation of lipid vesicles for partition coefficients

Multilamellar lipid vesicles (MLV)

Thin films were made in test tubes by the method of Bangham et al. (1965). 2 μmol of dioleoylphosphatidylcholine (DOPC) (or 2 mg of extracted lipids, or cholesterol/2 μmol of DOPC in various mole ratios) dissolved in 19/1 chloroform/methanol was dried under a stream of nitrogen while being vortexed to form a thin film. The film was kept under vacuum for 30 min; then 1 ml of buffer (150 mM NaCl, 10 mM Tris, pH 7.0) was added while the tube was vortexed to form a suspension of vesicles. 50 μl of this suspension was used per sample for $K_{p[mem]}$ measurements.

Unilamellar lipid vesicles (ULV)

ULV were made from MLV using the Lipo-So-Fast apparatus (MM Developments, Toronto, Ontario, Canada). 1 ml at a time of the MLV suspension was passed back and forth 13 times through a 100 nm filter and expelled after the 14th pass (a total of 27 passes through the filter). The suspension of vesicles resulting from this procedure is a mixture containing some bi-, tri-, and polylamellar vesicles as well as ULV (Rhodes et al., 1992). However, it approximates homogeneous ULV closely enough that any significant differences in partition coefficients from those determined in MLV are observable.

Membrane/buffer partition coefficients ($K_{p[mem]}$): association and dissociation rates of MK-801 from membranes

Details of the methods for these related experiments have been previously described (Herbette et al., 1989). Brief summaries are given below.

$K_{p[mem]}$

$K_{p[mem]}$s of MK-801 into membranes were measured by vacuum filtration (Brandel MR-48R cell harvester, Biomedical Research and Development Laboratories, Gaithersburg, MD) using [^{3}H]MK-801. The amount of membrane phospholipid retained on the filter during filtration was measured by means of vesicles radiolabeled with [^{14}C]DOPC or [^{3}H]cholesterol. Cholesterol is retained proportionately to phospholipid, and retention does not change with cholesterol content (Rhodes et al., 1992). Membrane/buffer partition coefficients ($K_{p[mem]}$) were calculated using the following equation: $K_{p[mem]}$ = (g of MK-801 bound to membrane/g of membrane phospholipid)/(g of MK-801 in filtrate/g of buffer).

Association

Nonspecific binding rates of [^{3}H]MK-801 to model and native membrane lipid vesicles were measured at 25°C at various time points from 15 s to 90 min. Solutions of [^{3}H]MK-801 (1.5 nM) and 12.5 μg/ml membrane in a pH 7.3 buffer (10 mM Tris, 150 mM NaCl) were filtered through glass fiber filters on a Brandel cell harvester. Because we were measuring nonspecific rather than specific binding of MK-801, the filters were not washed after filtration but were immediately counted for MK-801. Reaction mixtures containing MK-801 but no membrane were used to measure MK-801 binding directly to the filters. The number of radioactive counts of MK-801 detected on the filters was used, after correction for binding directly to the filters, to represent the amount of MK-801 bound to membranes. This number and the number of grams of lipid recovered on the filters were used for calculations. The amount of [^{3}H]MK-801 remaining in the filtrate was determined by subtraction.

Dissociation

Mixtures containing [^{3}H]MK-801 (1.5 nM) and 12.5 μg/ml membranes in a pH 7.3 buffer (10 mM Tris, 150 mM NaCl) were filtered through Whatman GF/C glass fiber filters on a Brandel cell harvester. The filters were not washed after filtration. Control reaction mixtures contained radiolabeled MK-801 but no membranes. Filters with membranes and control filters (no membrane) were immediately counted for radioactivity to find the maximal binding of MK-801 to membranes. The remaining filters were placed in a beaker containing buffer at 25°C. At appropriate time intervals, filters with and without membranes were taken out of the buffer and assayed for radioactivity. The percent of [^{3}H]MK-801 remaining bound to membranes at each time point was calculated.

Measurement of change in cooperative unit size

Sample preparation

Multilamellar vesicles of DMPC were produced by drying 100 μl aliquots of a 0.03 M solution of lipid in chloroform to a thin film by evaporation under N_2, with the residual solvent being removed by overnight evacuation. The lipid was then rehydrated for 10 min at 50°C with 100 μl of sample buffer (0.05 mM HEPES, 150 mM NaCl, pH 7.0) and vortexed for 1 min

to produce vesicles. For drug-containing samples, either 5.8 or 2.9 μl of 0.03 M drug solution (to obtain drug/lipid mole ratios of 1/17 or 1/35) was added to the lipid being dried down, with vesicles being produced as described above.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was carried out on drug-containing and control multilamellar vesicles. 15 μl aliquots of vesicles containing 300 μg of lipid were placed in DSC sample pans and hermetically sealed. A TA Instruments DSC 2910 Differential Scanning Calorimeter (Wilmington, DE) calibrated with an indium standard was used for data collection. A scan rate of 2°C/min starting from 6°C and going to 29.5°C was used for experiments. The reference was 15 μl of sample buffer. TA Instruments Thermal Analyst 2000 software was used for data analysis. The change in cooperative unit size was determined by the following formula:

$$\Delta CUS = \left(\frac{\Delta H_{vh}}{\Delta H * Fw}\right)_{drug} - \left(\frac{\Delta H_{vh}}{\Delta H * Fw}\right)_{control},$$

where ΔCUS is the change in the cooperative unit size, ΔH_{vh} is the van't Hoff enthalpy, ΔH is the calorimetric enthalpy, and Fw is the formula weight of the lipid undergoing transition.

X-ray diffraction

Sample preparation

Multilamellar vesicles were prepared as described above for $K_{p[mem]}$, using a ratio of 250 μg of phospholipid/50 μl of buffer. The buffer used was 0.5 mM HEPES, 2.0 mM NaCl, pH 7.3. When drug-containing samples were being made, the buffer contained sufficient MK-801 hydrogen maleate (Research Biochemicals Inc., Natick, MA) to yield a final MK-801/phospholipid mole ratio of 1/60 or 1/45. Lipid multibilayer samples for x-ray diffraction were made by the "spin-dry" technique by a variation (Chester et al., 1987) of the method of Clark et al. (1980), in which the volatile fluids in the sample are evaporated completely by the centrifuge vacuum system during sedimentation as previously reported (Moring et al., 1991). Enough vesicle preparation to contain 250 μg of phospholipid was used per sample. The samples were simultaneously sedimented onto aluminum foil and dehydrated, then mounted on a support and allowed to rehydrate over a saturated solution of a salt selected to maintain a given state of hydration.

Data collection and analysis

Diffraction data were collected using an Elliot GX18 rotating anode microfocus generator (Marconi Avionics) supplying Cu Kα radiation (λ = 1.54 Å) in conjunction with a fixed-geometry beamline consisting of a single Franks mirror providing line focus at the detection plane, vertical and horizontal limiting slits, a nickel filter, the curved specimen, a tungsten beamstop, and electronic detection. The beam height at the specimen was ~1 mm. Data collection was carried out with a Braun one-dimensional, position-sensitive proportional counter (Innovative Technology, Inc., Newburyport, MA). Data were transferred to a VAX 8200 (Digital Equipment Corporation, Maynard, MA) for reduction and analysis. Phases were determined by means of a swelling series. Perturbations caused by the presence of MK-801 were determined using electron density differences in conjunction with autocorrelation analysis (Young et al., 1992).

RESULTS

$K_{p[mem]}$ in 0.6 cholesterol/DOPC (mole ratio) MLV did not vary significantly over a range of MK-801 concentrations from 1.5×10^{-11} M to 4.5×10^{-9} M (Table 1). Thus, the nonspecific binding was not saturable over this concentration range. A physiologically reasonable MK-801 concentration of 1.5 nM, which is near the dissociation constant for specific binding (K_d) of MK-801, was chosen at which to conduct further experiments.

TABLE 1 $K_{p[mem]}$ of MK-801 in model membrane vesicles (±SD)

[MK-801] (M)	$K_{p[mem]}$ in MLV (cholesterol/DOPC mole ratio = 0.6)
1.5×10^{-11}	845 (±435)
4.5×10^{-11}	913 (±69)
1.5×10^{-10}	740 (±403)
4.5×10^{-10}	715 (±355)
1.5×10^{-9}	661 (±264)
4.5×10^{-9}	790 (±86)

Increasing the cholesterol/DOPC mole ratio markedly decreased $K_{p[mem]}$ in a concentration-dependent manner, indicating that MK-801 and cholesterol compete for the same space in the lipid bilayer (Table 2). The amount of DOPC was kept constant throughout; the additional cholesterol increased the total amount of lipid per sample. Our $K_{p[mem]}$ calculations have been carried out on the basis of the amount of phospholipid in the bilayer; cholesterol content was ignored. However, the effect of including cholesterol as well as phospholipid in the total lipid used in the $K_{p[mem]}$ calculations would tend to make $K_{p[mem]}$ even lower than the values reported here.

$K_{p[mem]}$ varied considerably among MLV of different lipid systems (Table 3). The cholesterol/phospholipid mole ratio of SNM lipid extract is about 0.6; enough cholesterol was added to the other systems to yield that mole ratio. The lipid systems that contain diverse lipid headgroups and/or diverse acyl chains (i.e., SNM lipid extract and BPC) yielded higher $K_{p[mem]}$s for MK-801 in MLV than did the more homogeneous systems. In every case, $K_{p[mem]}$ was significantly higher in MLV than in ULV. In fact, the differences among the $K_{p[mem]}$s for the various lipid systems disappeared when ULV were used. Trapping of MK-801 between the layers of MLV, possibly by a pH gradient mechanism (Cullis et al., 1991) and/or a difference in radius of curvature between MLV and ULV might be responsible for the higher $K_{p[mem]}$s in MLV. The diameter of MLV made by our method is 100–500 nm; that of ULV is ~100 nm (Rhodes et al., 1992).

In MLV, $K_{p[mem]}$ may vary with pH, especially for MK-801, the nitrogen atom of which can be protonated and thus would carry a charge. Our data showed a decrease in $K_{p[mem]}$

TABLE 2 Effect of cholesterol content on $K_{p[mem]}$ of MK-801 (1.5 nM) in cholesterol/DOPC vesicles

Mole ratio cholesterol/DOPC	$K_{p[mem]}$ (±SD) in MLV	$K_{p[mem]}$ (±SD) in ULV
0.00	3374 (±253)	1137 (±320)
0.15	1040 (±53)	1080 (±38)
0.30	1156 (±129)	862 (±219)
0.45	963 (±166)	802 (±72)
0.60	804 (±82)	582 (±138)

TABLE 3 $K_{p[mem]}$ of MK-801 (1.5 nM) in vesicles of various lipids + cholesterol

Lipid system (cholesterol/lipid mole ratio = 0.6)	$K_{p[mem]}$ (±SD) (MLV)	$K_{p[mem]}$ (±SD) (ULV)
SNM lipid extract (no additional cholesterol)	6879 (±947)	485 (±99)
Bovine phosphatidylcholine (BPC)	2704 (±961)	364 (±49)
Dioleoylphosphatidylcholine (DOPC)	746 (±65)	421 (±25)

TABLE 4 Effect of pH on $K_{p[mem]}$ in MLV (mole ratio cholesterol/DOPC = 0.6)

pH	$K_{p[mem]}$
7.0	523 (±164)
8.0	211 (±251)
9.0	293 (±132)
10.0	160 (±66)
11.0	132 (±292)

at higher pH in MLV (Table 4). However, all of our $K_{p[mem]}$s were determined at pH 7.0, at which MK-801 is probably highly protonated.

MK-801 is not sufficiently water-soluble that stock solutions can be made in buffer. The [^{3}H]MK-801 stock solutions were made up in ethanol; therefore, some ethanol was present in all experiments. Addition of ethanol to SNM preparations (with 1.5 nM MK-801) in concentrations as high as 0.11 M in addition to the ethanol present in the stock solutions did not change $K_{p[mem]}$ (data not shown). The highest concentration of ethanol in any incubation mixture (without additional ethanol) was 0.064 M, and the lowest was 3.5×10^{-4} M; the usual concentration was 8.3×10^{-4} M (for [MK-801] = 1.5 nM). Thus, ethanol did not appear to alter $K_{p[mem]}$ in these experiments. These data indicate that nonspecific binding of MK-801 either is not an interaction with the membrane surface or is a surface interaction that is simply unaffected by the presence of ethanol with its attendant effects on hydrogen bonding in the hydrated region.

To measure $K_{p[mem]}$ of MK-801 in intact biological membranes, we used a synaptoneurosome (SNM) preparation. Table 5 shows that $K_{p[mem]}$ was constant over a wide range of MK-801 concentrations, indicating that a true partition coefficient had been measured; that is, no observable specific binding had occurred. Nevertheless, an attempt was made to use literature values of the dissociation constant (K_d) and the maximum number of binding sites (B_{max}) to correct for specific binding. The K_d of MK-801 in rat cortical membranes has been measured at 37.2 ± 2.7 nM; the maximum number of binding sites (B_{max}) was 0.825 ± 0.102 pmol/mg protein (Wong and Woodruff, 1986). This experiment appears to have been conducted in the absence of exogenous NMDA receptor agonists. Other experiments using rat cortex, conducted in the presence of the agonists glutamate and glycine, yield $K_d \approx$ 1–4 nM, although B_{max} is not significantly different (Kloog et al., 1988; Steele et al., 1991). The correction caused $K_{p[mem]}$ to go to zero at higher MK-801 concentrations, a further indication that no specific binding occurred in the SNM preparation. Thus, the true $K_{p[mem]}$ is close to the uncorrected value. $K_{p[mem]}$ in SNM was about the same as in ULV of model membrane systems containing 0.6 cholesterol/DOPC (Table 2).

Change in cooperative unit size

Differential scanning calorimetry was used to assess the effect of various concentrations of MK-801 on membrane disorder by measuring the change in the cooperative unit size (ΔCUS) of dimyristoylphosphatidylcholine (DMPC) bilayers. The decrease in the main phase transition temperature (ΔT_m) was also measured. The effect of MK-801 was compared with those of the 1,4-dihydropyridine calcium channel antagonists nifedipine and nimodipine (Table 6). A decrease in *CUS* indicates an increase in bilayer disorder. All three drugs caused decreases in CUS and T_m in a concentration-dependent manner. Both of the dihydropyridines caused larger changes than did MK-801. Thus, MK-801 perturbed the hydrocarbon core of the bilayer, but less than the dihydropyridines did. This indicates a relatively weak interaction of MK-801 with the membrane.

Kinetics of MK-801 association with and dissociation from membranes

Association of MK-801 with model membranes (cholesterol/DOPC = 0.6) was rapid (Fig. 2 *A*), being complete in less than 5 min. Similarly, dissociation was complete within 10 min (Fig. 3 *A*). Although such fast association of drugs with membranes is common, the dissociation rate of MK-801 is

TABLE 5 $K_{p[mem]}$ of MK-801 in intact synaptoneurosomes

[MK-801] (M)	Proportion of receptors occupied*	$K_{p[mem]}$ (including specific binding)	$K_{p[mem]}$ corrected for specific binding
4.5×10^{-11}	1.2×10^{-3} ($\pm 8.8 \times 10^{-5}$)	247 (±200)	209 (±144)
1.5×10^{-10}	4.0×10^{-3} ($\pm 2.9 \times 10^{-4}$)	430 (±129)	367 (±131)
4.5×10^{-10}	0.012 ($\pm 8.7 \times 10^{-4}$)	363 (±115)	326 (±137)
1.5×10^{-9}	0.043 ($\pm 3.1 \times 10^{-3}$)	428 (±117)	365 (±118)
4.5×10^{-9}	0.11 ($\pm 7.8 \times 10^{-3}$)	386 (±48)	224 (±52)
1.5×10^{-8}	0.29 (±0.029)	354 (±194)	0
4.5×10^{-8}	0.55 (±0.040)	191 (±160)	0
1.5×10^{-7}	0.80 (±0.058)	250 (±82)	0

* Receptor occupancy calculated according to $Y = (1/K_D)[A](1-Y)$, where Y = proportion of receptors occupied; $[A]$ = concentration of MK-801 (M); K_D = dissociation constant (M); K_D = 37.2 nM, B_{max} = 0.825 pmol/mg protein (Wong et al., 1986).

TABLE 6 Change in cooperative unit size of DMPC caused by presence of MK-801

	MK-801		Nifedipine		Nimodipine	
Drug/DMPC mole ratio	1:35	1:17	1:35	1:17	1:35	1:17
ΔT_m (°C)*	−0.13	−0.71	−1.07	−1.42	−0.76	−1.76
Δ Cooperative unit size (molecules)	−52	−92	−111	−168	−79	−132

* T_m = temperature of main phase transition (°C).
Control DMPC: T_m = 23.05°C; cooperative unit size = 220 molecules.

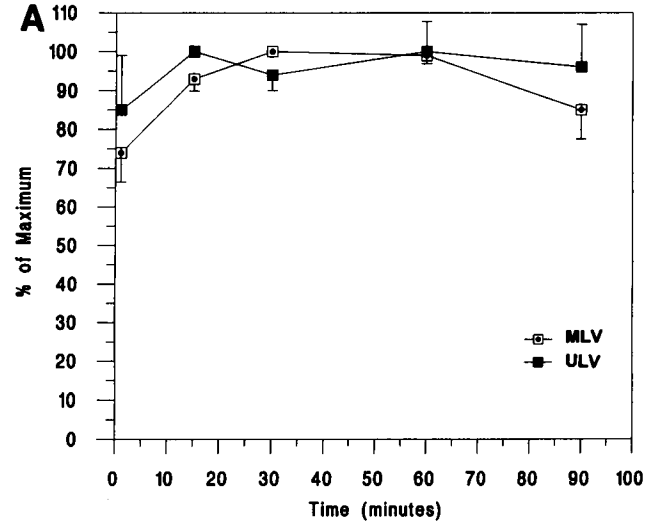


FIGURE 2 Kinetics of association ("Wash-in" studies). (*A*) Association of MK-801 with cholesterol/DOPC vesicles. (□) MLV; (■), ULV; phospholipid concentration = 12.5 μg/ml, cholesterol/DOPC mole ratio = 0.6, [MK-801] = 1.5 nM. (*B*) Association of MK-801 with intact SNM (not lipid extract). [MK-801] = 1.5 nM. Wash-in was rapid in both cases, but more MK-801 entered the cholesterol/DOPC vesicles.

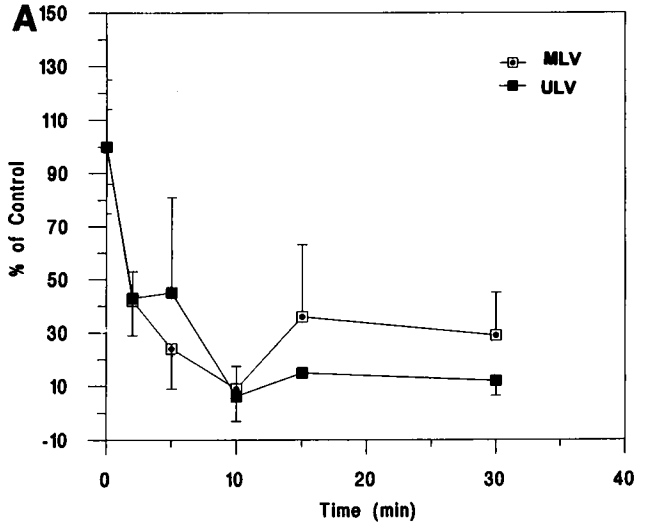


FIGURE 3 Kinetics of dissociation ("Washout" studies). (*A*) Dissociation of MK-801 from cholesterol/DOPC vesicles. (□), MLV; (■), ULV; phospholipid concentration = 12.5 μg/ml, cholesterol/DOPC mole ratio = 0.6, [MK-801] = 1.5 nM. (*B*) Dissociation of MK-801 from intact SNM (not lipid extract). [MK-801] = 1.5 nM. Most of the MK-801 washed out of cholesterol/DOPC vesicles within 5 min. Washout from synaptoneurosomes was slower, possibly because of their protein content.

relatively fast compared with some other drugs tested in this laboratory (Rhodes et al., 1992; Herbette et al., 1986). The rapid dissociation suggests that although the drug is only slightly water-soluble, its location in the membrane is probably within or very close to the hydrated region, easily accessible to the interbilayer water space. Similar experiments

were carried out using intact SNM (not vesicles made from lipid extract). Figs. 2 *B* and 3 *B* show that rates of MK-801's association with and dissociation from SNM are similarly fast.

X-ray diffraction

Three cholesterol/DOPC mole ratios, 0.0, 0.15, and 0.60, were used in the model membranes in these experiments. The highest was chosen to match the experimentally determined mole ratio in our SNM preparations, and the lowest was used to maximize the amount of MK-801 entering the bilayer. Most experiments were carried out at a high state of bilayer hydration, nominally 96% relative humidity, to approximate the high hydration of membranes in vivo. Experiments were conducted at MK-801/DOPC ratios of 1/60 and 1/45 so that the bilayers examined would be identical in structure except for the amount of MK-801 present. Under these conditions, the difference electron density profile should represent only differences caused by the presence of more MK-801. The aim was to find the actual equilibrium nonspecific binding location of MK-801 by using electron density differences and the autocorrelation function.

Diffraction patterns for cholesterol/DOPC multilayer samples containing MK-801 typically had four or five orders at a high hydration state of the membrane, depending on the amount of cholesterol present. Fig. 4 shows a typical pattern. MK-801 perturbed cholesterol/DOPC bilayers in a concentration-dependent manner. These perturbations, although sometimes small and variable in size, occurred consistently over several experiments.

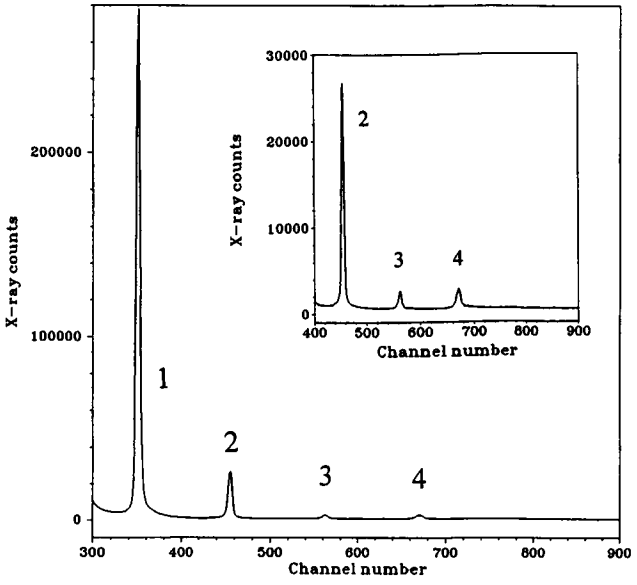


FIGURE 4 Diffraction pattern from a cholesterol/DOPC multibilayer sample, mole ratio = 0.6, MK-801/DOPC = 1/45, d = 57.8 Å. (*inset*) Orders 2, 3, and 4 enlarged for clarity. Data are shown before background subtraction and Lorentz correction. The relative humidity was nominally 96%. The highest cholesterol/DOPC ratio consistently yielded four orders. Lower ratios gave four or five orders.

High hydration, high cholesterol

Most of our x-ray analysis was conducted using samples having cholesterol/DOPC = 0.6 and high hydration. These samples gave the most consistent data and are most relevant to the composition and high in vivo hydration state of the brain membranes of interest. In these samples, the cholesterol/DOPC bilayer was in the L_α phase with both 1/60 and 1/45 MK-801/DOPC (Fig. 5). Increasing the amount of MK-801 in the bilayer increased the degree of bilayer melting, as indicated by a less pronounced curve at the acyl chain region of the profile. It also increased the electron density in the glycerol back bone-upper acyl chain region somewhat (Fig. 5). At 0.6 cholesterol/DOPC, most control samples (without MK-801) showed evidence of phase separation. Thus, MK-801 in these amounts tended to prevent phase separation.

Autocorrelation analysis (Young et al., 1992) was used to model the perturbation caused by MK-801 in these higher cholesterol samples. The bilayer model was built from two Gaussians centered at ±21.37 Å representing the headgroups and another of negative amplitude centered at 0 Å representing the methyl trough. A perturbation centered at 16 Å from the center of the bilayer successfully modeled the small decrease in headgroup spacing and the slight widening of the headgroups observed at the higher MK-801 concentration. The other major perturbation, a negative difference centered in the water space, was ignored in the model. The experimentally determined multilayer difference autocorrelation

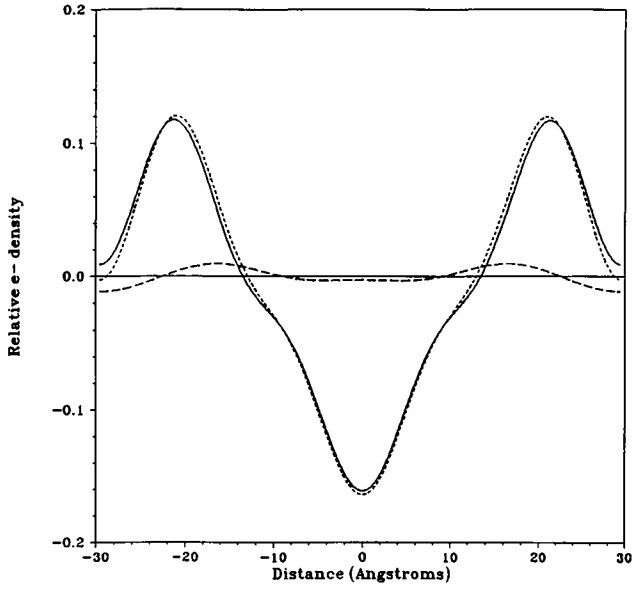
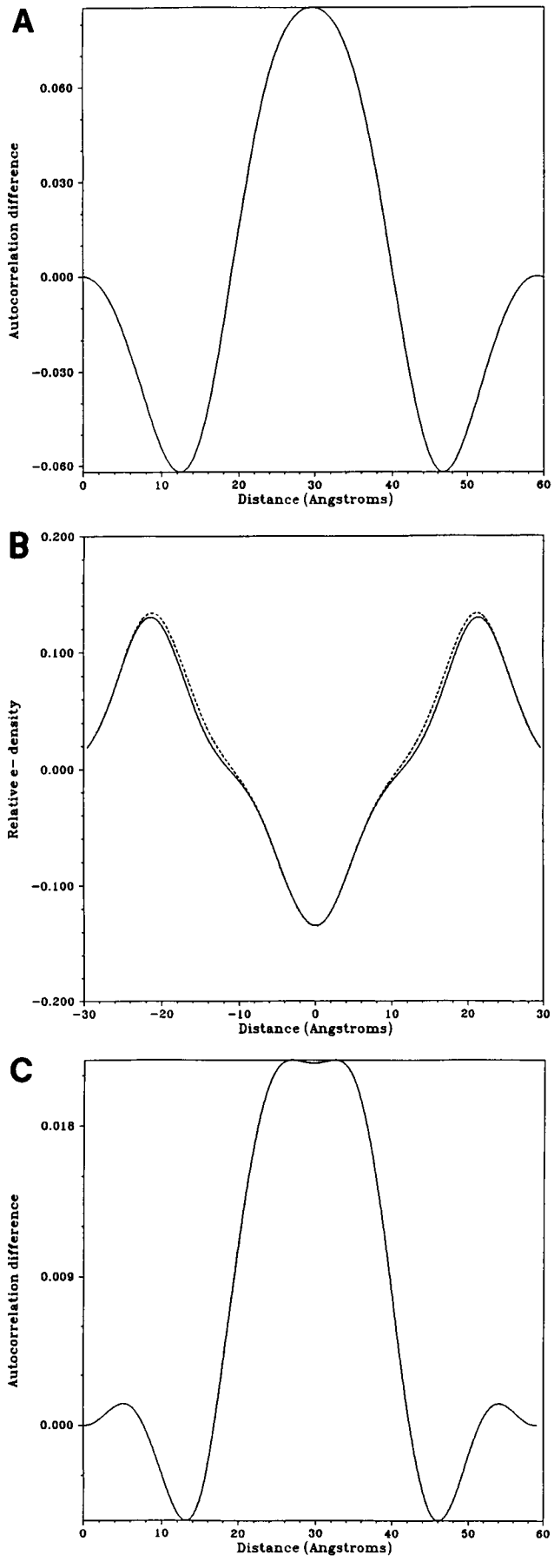


FIGURE 5 Electron density profiles at high hydration and high cholesterol content (96%, 0.6). Continuous structure factors were calculated and sampled at d = 59.1 Å Solid profile, MK-801/DOPC = 1/60, original d = 59.1 Å. Dotted profile, MK-801/DOPC = 1/45, original d = 58.5 Å. (——) Difference. In this and some other experiments, the headgroups were moved 0.8 Å closer together with more MK-801 present, but sometimes the distance between headgroups was unchanged. MK-801 appeared to stabilize the bilayer. Diffraction from control samples (without MK-801) at this cholesterol/DOPC mole ratio usually showed phase separation.



function and the one calculated from the model are shown in Fig. 6. The model, using the single perturbation, reproduces the major features of the experimental difference autocorrelation function. The perturbation could be shifted no further than ±2 Å from its 16 Å location without serious disagreement between the modeled and experimental difference autocorrelation functions. The perturbation at 16 Å in the electron density profile is likely to be caused by the actual presence of MK-801 at that location, but it is also possible that it results indirectly from the presence of MK-801 at some other location in the bilayer.

High hydration, intermediate cholesterol

The interaction of MK-801 with 0.15 cholesterol/DOPC was more complicated than its interaction with the higher cholesterol membrane. A typical set of electron density profiles is shown in Fig. 7. The control and MK-801/DOPC = 1/60 profiles were found to be indistinguishable, whereas the MK-801/DOPC = 1/45 profile represented a different structure. At this cholesterol/DOPC ratio, the 1/60 and 1/45 structures were not isomorphous, so the electron density differences could not be interpreted as being caused by MK-801. As in experiments using high cholesterol membranes, increasing MK-801 content resulted in a more poorly defined glycerol backbone-acyl chain region of the profile, indicating progressive degrees of bilayer melting.

High hydration, no cholesterol

According to the $K_{p[mem]}$ values, more MK-801 should enter a pure DOPC membrane than a cholesterol-containing DOPC membrane. Higher MK-801 content might help determine the actual nonspecific binding location of MK-801 in the bilayer. However, the interaction of MK-801 with pure DOPC turned out to be more complex than that with 0.6 cholesterol/DOPC. 1/60 MK-801/DOPC caused an electron density increase at ~9 Å from the bilayer center and a decrease at the methyl trough, as compared with control (Fig. 8). This yielded a better defined acyl chain-glycerol backbone region of the electron density profile and probably indicates that MK-801 is entering the acyl chain region at about the position where cholesterol would be located. In most samples, 1/45 MK-801 caused an electron density increase at the upper acyl chain region similar to that caused by 1/60 MK-801/DOPC. This increase was accompanied by an increase at the methyl trough region. In most experiments, both

FIGURE 6 Autocorrelation analysis. (*A*). Experimentally determined difference autocorrelation function. This difference autocorrelation function was calculated from the x-ray intensity data used to produce the electron density profiles shown in Fig. 5 (high membrane hydration, cholesterol/DOPC = 0.6, MK-801/DOPC = 1/45 and 1/60). (*B*) Model electron density profiles. These electron density profiles model the electron density profiles shown in Fig. 5. Construction of the model is explained in the text. (*C*) Difference autocorrelation function calculated from the models. This function reproduces the major features of the experimentally determined function in Fig. 6 *A*.

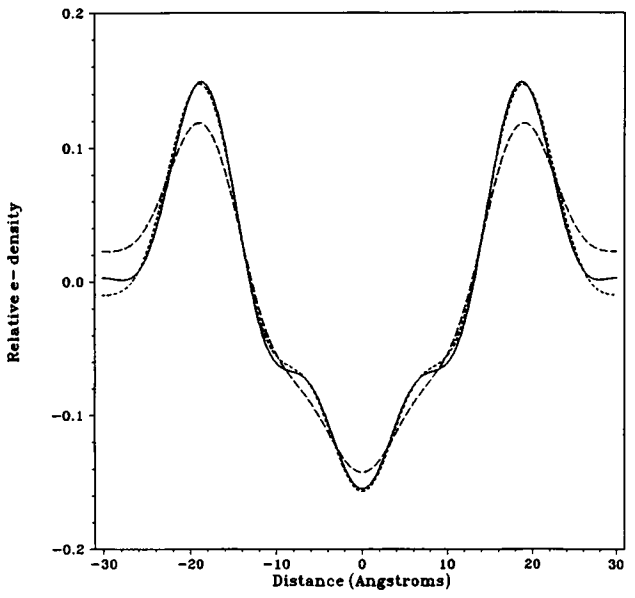


FIGURE 7 Electron density profiles at high hydration and low cholesterol content. (96%, cholesterol/DOPC = 0.15). Continuous structure factors were calculated and sampled at d = 60.4 Å. Solid profile, control (no MK-801), original d = 59.8 Å. Dotted profile, MK-801/DOPC = 1/60, original d = 59.7 Å. Dashed profile, MK-801/DOPC = 1/45, original d = 60.4 Å. Repetitions of this experiment showed that the control and MK-801/DOPC = 1/60 are not different within the experimental error and that 1/45 MK-801/DOPC is not isomorphous to the other two profiles.

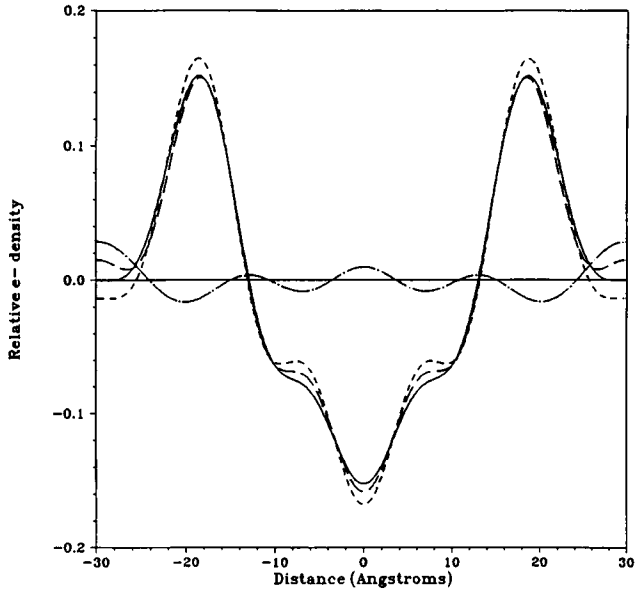


FIGURE 8 Electron density profiles at high hydration, no cholesterol present. Continuous structure factors were calculated and sampled at d = 59.7 Å Solid profile, control (no MK-801), original d = 57.1 Å. Dotted profile, MK-801/DOPC = 1/60, original d = 57.9 Å. Dashed profile, MK-801/DOPC = 1/45, original d = 59.5 Å. Dashed-dotted line, difference. The electron density differences in the absence of cholesterol are complex, but the interpretation that some MK-801 may reach the bilayer center at 1/45 MK-801/DOPC is consistent with the results. At 1/60 acyl chain region density and headgroup density increase; at 1/45, methyl trough density increases. 1/45 MK-801/DOPC did not necessarily increase the state of melting of the bilayer over control; thus, MK-801 did not contribute significantly to bilayer disorder.

MK-801/DOPC ratios appeared to cause ordering of the bilayer, with 1/60 > 1/45. Occasionally, however, 1/45 MK-801/DOPC caused bilayer melting instead. The higher concentration of MK-801 always caused an increase in electron density in the methyl trough region over that of the lower concentration, which probably indicates that at 1/45, some MK-801 is entering the methyl trough region.

Lower hydration states

Some experiments were carried out at lower bilayer hydration states for the purpose of clarifying the MK-801-membrane interaction. Although the interactions were better defined at lower hydration, they were different from those at higher hydration and therefore were not useful for interpreting the higher hydration state. Nevertheless, some observations are of interest. At a lower hydration state (nominally 84%, cholesterol/DOPC = 0.6), neither MK-801 concentration increased bilayer melting. The perturbations of the bilayer by MK-801 were closer to the bilayer center. At 72% (cholesterol/DOPC = 0.15), the perturbation caused by MK-801 was ~7 Å from the bilayer center, probably because of the lack of excess water in the hydrated region. Only the higher MK-801 concentration caused bilayer melting.

DISCUSSION

The chemical structure of MK-801 indicates a bulky, rigid molecule with hydrophobic ring systems and a basic nitrogen atom that is likely to be protonated at physiological pH. Its $K_{p[mem]}$, although relatively low, shows that MK-801 enters lipid bilayers in significant amounts. Its nonspecific equilibrium binding location in a bilayer of given composition is likely to depend on the balance between the charge on the protonated nitrogen atom in the central ring system and the hydrophobic character of the rings. The decrease of $K_{p[mem]}$ with increasing pH indicates that the protonation and hydrogen bonding capacity of MK-801 are more important determiners of the extent of its nonspecific membrane binding than its hydrophobic character.

At least two explanations are possible for the decrease in $K_{p[mem]}$ with increasing membrane cholesterol content. 1) Cholesterol in relatively low concentrations tends to order the bilayer. More space for drug molecules is likely to be available in a disordered bilayer (King et al., 1985). 2) MK-801 is a rigid, bulky molecule that appears to position itself preferentially in a region of the membrane in which cholesterol is located. The cholesterol may be directly displacing MK-801 from its preferred location in the bilayer.

Ethanol did not affect $K_{p[mem]}$ of MK-801. The lower ethanol concentrations in these experiments are physiologically relevant, although the highest concentration used (0.11 M) represents a dangerous, possibly lethal concentration in the blood in vivo. Ethanol is preferentially located in the hydrated region of the membrane rather than in the hydrophobic

core (Herbette et al., 1985). That in vitro ethanol exposure failed to alter $K_{p[mem]}$ of MK-801 indicates that even at high hydration states and high cholesterol concentration, this drug may be somewhat deeper within the hydrophobic region of the membrane than the perturbation at ~16 Å from the bilayer center that we modeled in our autocorrelation studies.

Electron density profiles were determined at high hydration because this state is more physiologically relevant than a lower hydration state. Thus, the in vivo perturbation of membranes by MK-801 is most likely to be centered in the glycerol backbone region. Whether the perturbation modeled at 16 Å represents the average equilibrium nonspecific binding location of actual molecules of MK-801, or is instead the major perturbation resulting from molecules at another location is uncertain. The crystal structure of a compound closely related to MK-801 has been determined (Leeson et al., 1990a). Its chemical structure is the same as that of MK-801 except that there is one more carbon atom, with two hydrogens, in the central ring system. The crystal structure parameters and chemical formula ($C_{17}H_{17}N$) of this compound were used to calculate its electron density (0.39 $e^{-}Å^{-3}$), which approximates the electron density for MK-801 ($C_{16}H_{15}N$ without its maleate counter ion). Average electron densities for the phosphate headgroup region (0.43 $e^{-}Å^{-3}$), fatty acyl chain region (0.26 $e^{-}Å^{-3}$), and terminal methyl region (0.196 $e^{-}Å^{-3}$) of a DPPC bilayer have been calculated (Trumbore et al., 1988). Using these DPPC electron densities to approximate those of DOPC bilayer regions, we find that the contrast of MK-801 with the headgroup is insufficient to show the drug directly if it is in the headgroup region. Cholesterol is located in the glycerol backbone and upper acyl chain regions of the bilayer (Franks, 1976), where its presence tends to increase the electron density. The electron density caused by MK-801 would probably be directly observable as an increase only in the acyl chain or methyl trough regions, and perhaps not even in the upper acyl chain region of a cholesterol-containing bilayer.

The binding of MK-801 to its receptor is slow in the absence of NMDA receptor agonists ($t_{½}$ = 70 min), but faster when glutamate and glycine are present ($t_{½}$ = 5–8 min) (Kloog et al., 1988). (These agonists increase the rates of association and dissociation, but not K_d.) Thus, the nonspecific association of MK-801 with membranes is on a time scale similar to that of receptor binding in the presence of agonists. The perturbation found at 16 Å is not as deep in the membrane as the putative location of the MK-801 binding site, which is thought to be within the hydrophobic region of the membrane rather than in the hydrated headgroup region (Monaghan et al., 1989; Winger, 1987; Sakurada et al., 1993). Approach to the specific binding site by first partitioning into the membrane and then diffusing laterally toward the site has been proposed for some drugs (Hille, 1977; Herbette et al., 1986; Herbette et al., 1989; Rhodes et al., 1985). The apparent requirement of an open channel for MK-801 binding and the location of the major perturbation in the glycerol backbone-upper acyl chain region in the absence of a deeper perturbation are evidence that a route through the membrane to the binding site is unlikely for this drug. However, because specific binding does take place, although extremely slowly, in the absence of NMDA receptor agonists, a membrane route might be possible when the channel is closed. If the receptor is located in a cholesterol-poor membrane domain, MK-801 might penetrate the membrane more extensively and deeply in the vicinity of the receptor, making a route through the membrane more likely. In any case, we speculate that MK-801 in the membrane, nonspecifically bound in the glycerol backbone region, might create a pool of MK-801 in equilibrium with drug in the interbilayer water space. Such a reserve pool might lengthen the time that the drug is available near the specific binding site and/or increase the amount available.

The change in glycerol backbone-acyl chain region shape, increase in bilayer disorder, and lack of extensive alterations in the acyl chain-methyl trough region of the electron density profile (cholesterol/DOPC = 0.6) are consistent with, but not proof of, a glycerol backbone region location of MK-801. Furthermore, the molecule contains a basic nitrogen atom that is probably protonated at pH 7.0, and that can hydrogen-bond (Leeson et al., 1990b). Although the hydrophobicity of its aromatic systems would tend to place MK-801 in the acyl chain region of the bilayer, the presence of a positive charge and hydrogen bonding capability would tend to place the drug in the hydrated region. We speculate that the MK-801 molecule, like cholesterol, is preferentially located at the interface between the hydrated headgroup region and the hydrophobic acyl chain region, with the nitrogen atom in the hydrated area and the ring system spread out below the interface in the hydrophobic upper acyl chain region. Sterically, such an orientation of MK-801 would be unfavorable, which might contribute to its surprisingly low $K_{p[mem]}$. The actual equilibrium nonspecific binding location and membrane orientation could probably be determined by neutron diffraction experiments using deuterated MK-801. The interaction of MK-801 with cholesterol/DOPC membranes as determined by x-ray diffraction is variable depending on cholesterol content and membrane hydration state. Because of variations in lipid composition among tissues, particularly in cholesterol content, the interaction is also likely to be highly variable in vivo.

The time required for MK-801 to associate with or dissociate from the membrane is similar in MLV, ULV, and SNM. The fast kinetics are consistent with the relatively low $K_{p[mem]}$ (this work) and the quick onset and short-acting nature of MK-801 in vivo (Troupin et al., 1986; Vezzani et al., 1989). A high $K_{p[mem]}$ is often correlated with a slow rate of dissociation from the membrane, but not necessarily with a slow rate of association. Lacidipine, a 1,4-dihydropyridine calcium channel blocker with a very high partition coefficient and an equilibrium nonspecific binding location in the hydrocarbon core, has slow rates of association with and dissociation from membranes (Herbette et al., 1993). Lacidipine also has both a long onset time and long duration of action in vivo. Amiodarone, a long acting antiarrhythmic drug with an extremely high $K_{p[mem]}$ (Herbette et al., 1986;

Trumbore et al., 1988), has a slow rate of membrane dissociation (Herbette and Gruner, 1987), yet it associates with the membrane quickly (D. W. Chester, personal communication).

The major conclusions to be drawn from this work are as follows:

1) The fast rates of association and dissociation of MK-801 with model and native membranes in vitro are consistent with a quick onset and short duration of action in vivo.
2) Cholesterol in the membrane interferes in a concentration-dependent manner with partitioning of MK-801 into the bilayer.
3) The equilibrium nonspecific binding location of MK-801 in high cholesterol membranes is most likely to be in the hydrated glycerol backbone-headgroup region. In membranes of lower cholesterol content, MK-801 appears to penetrate more deeply.
4) A route for MK-801 through the membrane to its specific binding site is unlikely, except perhaps in the absence of receptor agonists. Nevertheless, MK-801 nonspecifically bound in the hydrated region or at the hydrophilic-hydrophobic interface might act as a reserve pool accessible to the specific binding site through the aqueous region.

We thank Mr. Robert Dring for assistance with the DSC experiments.
This work was supported by National Institute on Alcohol Abuse and Alcoholism #5 K 21 AA00126 and Heublein, Inc.

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