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AN EPR STUDY OF STRUCTURAL PERTURBATIONS INDUCED BY 3-METHYLINDOLE IN THE PROTEIN AND LIPID REGIONS OF ERYTHROCYTE MEMBRANES

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SUMMARY

3-Methylindole has been shown in previous work to cause pulmonary edema and emphysema in cattle and goats. In this paper, evidence is presented to show that 3-methylindole induces structural perturbations in bovine erythrocyte membranes. The structural perturbations which were induced as a function of 3-methylindole concentration in the membranes were measured by EPR using the attachment of a maleimide spin label to the sulfhydryl groups of membrane proteins and by intercalation of methyl-5- doxylstearate, methyl-12-doxylstearate, and methyl-16-doxylstearate into the lipid region. The EPR spectra of the maleimide spin-labeled membrane proteins became more immobilized as the concentration of 3-methylindole increased. The order parameter describing the EPR spectra of methyl-5-doxylstearate decreased from 0.69 to 0.55 as the concentration of 3-methylindole increased. The acyl chains in the region of the carbon 5 position were converted to a less ordered structure. The EPR-spectra of methyl-12-doxylstearate was a superposition representing at least three tumbling rates. As the concentration of 3-methylindole increased, the major fraction of the methyl-12-doxylstearate probes experienced an increase in tumbling rate and a smaller fraction is observed in a strongly immobilized state. The EPR spectra of methyl-16-doxylstearate were not perceptibly changed in the presence of 3-methylindole.

The concentration dependence suggests that 3-methylindole preferentially intercalates into the ordered region of the alkyl chains sampled by the methyl-5-doxylstearate. These results confirm that 3-methylindole induced structural changes at the molecular level.

INTRODUCTION

3-Methylindole is one of the main end products of tryptophan fermentation in the rumen [1]. Intraruminal doses and infusion of 3-methylindole induced acute pulmonary edema and emphysema in cattle and intraruminal doses were effective in

goats [2]. Since only intraruminal doses of tryptophan had previously been shown to cause pulmonary lesion [3], these results suggested that 3-methylindole is the causative agent in tryptophan-induced pulmonary edema and emphysema in cattle. The clinical signs and pulmonary lesions of this experimentally induced disease resemble those in naturally occurring acute pulmonary edema and emphysema in cattle [4].

The mechanism by which 3-methylindole causes pulmonary lesions in cattle and goats is unknown, but 3-methylindole can adversely affect biological membranes. High concentrations (500 μg/ml) of 3-methylindole can hemolyze erythrocytes [5], rupture ruminal protozoa, particularly holotrichs [6]. Injection of 3-methylindole into rabbit knee joints causes inflammation of intraarticular membranes resulting in arthritic joint damage [7]. The ability of 3-methylindole to disrupt biological membranes may be related to its lipophilic properties [8].

Tissue damage by 3-methylindole suggested an interaction with biological membranes, and the effect on artificial inverted lecithin micelles has been examined using NMR spectroscopy. 3-Methylindole intercalated between the lecithin fatty acid chains near the polar head with the hydrogen on position 1 of the indole ring forming a hydrogen bond with the oxygen on the phosphate. It was concluded that 3-methylindole interacted with lecithin, and reduced the water mobility in this system [9].

In this paper we describe the structural perturbations induced by 3-methylindole in the protein and lipid regions of bovine erythrocyte membranes. Spin-labeled erythrocytes have been used to study structural perturbations in membranes induced by phenothiazine derivatives [10] and benzyl alcohol [11]. The maleimide spin label reacts specifically with sulfhydryl groups on the erythrocyte membrane proteins [12]. Stearate spin labels provide information about the degree of molecular order in the lipid environment [13–16]. The choice of bovine erythrocytes provides continuity of species used in the pathological studies.

MATERIALS AND METHODS

Fresh bovine erythrocytes were obtained from 500 ml of citrated blood. The cells were packed at $700 \times g$ for 5 min and washed three times in 0.17 M NaCl. The ghosts were prepared by the method of Dodge et al. [17] (large scale preparation of hemoglobin free ghosts). The spin label used for labeling the membrane protein was *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)-maleimide (maleimide spin label). Methyl-5-doxylstearate, methyl-12-doxylstearate, and methyl-16-doxylstearate were used for labeling the membane lipids (Synvar, Palo Alto, Calif.). The methylstearate spin labels were purified by thin-layer chromatography on silica gel. 3-Methylindole was obtained from Sigma Chemical Co., St. Louis, Mo. The EPR spectrometer was a Varian E-9 X-band instrument.

The ghosts were incubated with 0.04 mg of spin label per ml of packed ghosts at 25 °C for 4 h. After labeling, the ghosts were washed three times with 0.05 M sodium phosphate buffer (pH 7.5) and resuspended in 0.17 M NaCl to give a 50 % (w/v) suspension.

10 mg of 3-methylindole were dissolved in 1 ml of chloroform in a 50-ml round bottom flask. The solvent was evaporated to dryness under reduced pressure, leaving the 3-methylindole evenly coated on the sides of the flask. The labeled ghosts

(22 ml) were then pipetted into the coated flask and incubated at 37 °C in a shaking water bath. Labeled control ghosts were incubated in a 50-ml round bottom flask without 3-methylindole. After incubation for 5, 15, 30, 60 and 120 min, 4-ml aliquots were removed and centrifuged at 34 500 × *g* for 10 min. 0.5 ml of packed ghosts were used for 3-methylindole quantitation, 0.2 ml of packed ghosts were used for protein determination, and 0.2 ml were used in the EPR spectrometer. Extraction and quantitation of 3-methylindole was done according to the method of Bradley and Carlson [18]. The same gas chromatography, conditions were used for analysis of methyl-12-doxylstearate. Total protein content of the ghosts was determined by the biuret reaction [19] since 3-methylindole will interfere with the Lowry method.

To test for hydrolysis of methylstearate, the supernatant from a spin-labeled ghost suspension was acidified, extracted with chloroform, and the extract was applied to silica gel G thin-layer plates. The chromatograms were developed in hexane/diethyl ether (7:3, v/v), and the spots were visualized and compared with standards using iodine spray.

RESULTS

The effect of 3-methylindole on ghost membrane lipid

The intercalation of 3-methylindole into the membrane alters the fluidity gradient as monitored by spin-labeled stearate probes. The molecular features of the disruption of the bilayer are studied as a function of concentration of 3-methylindole in the membrane and as a function of the depth of the oxazollidine ring in the lipid bilayer.

The EPR spectra of the methyl-5-doxylstearate-labeled membranes are characteristic of rapid anisotropic tumbling about the long axis of an extended acyl chain (see refs 14–16). The uptake of 3-methylindole by these membranes and the changes in the order parameter as a function of 3-methylindole incubation time are shown in Fig. 1. By projection, the dependence of the order parameter on the concentration of 3-methylindole is obtained. The order parameter provides a measure of the local

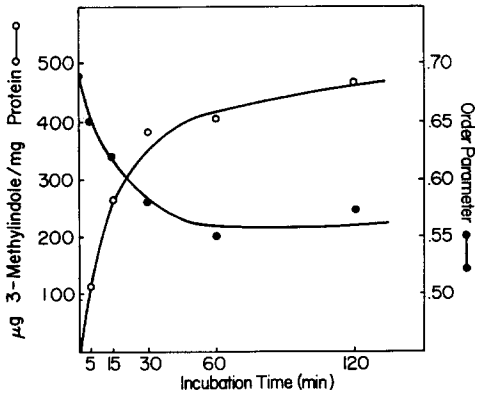

Fig. 1. The uptake of 3-methylindole by bovine erythrocyte ghost membranes labeled with methyl-5-doxylstearate and the order parameter, *S*, as a function of incubation time with 3-methylindole.

ordering of acyl chains of the phospholipids; the extent of local ordering decreases as the order parameter decreases from 1 to 0. We observe a monotonic relationship between the concentration of 3-methylindole in the membrane and the decrease in the order parameter. Both the time course for the 3-methylindole and the order parameter approach limiting values concurrently. The interpretation of the change in the order parameter is that the acyl chains in the region of the carbon 5 position are converted to a less ordered structure.

The EPR spectra of erythrocyte membranes labeled with methyl-12-doxylstearate are compared in Fig. 2 as a function of 3-methylindole concentration in the membrane. The characterization of the structural changes cannot be reduced to a simple parameter (such as a correlation time) because of the complexity of the spectra. In the absence of 3-methylindole, the EPR spectrum (Fig. 2A) is the superposition of an intermediately immobilized fraction (II), a strongly immobilized fraction (I) and a weakly immobilized fraction (III), but Fraction II is largest of the three. As the concentration of 3-methylindole increases, significant line shape changes occur. The majority of the spin labels in Figs 2C to 2E have an increased tumbling rate while a strongly immobilized fraction (I) is also present. The spectral changes of methyl-5-doxyl stearate appear to exhibit more pronounced changes at lower concentrations than that of the methyl-12-doxylstearate. The increase in tumbling rate of a significant fraction of the spin label probes suggests that 3-methylindole perturbs the structure of the membrane at the carbon 12 position.

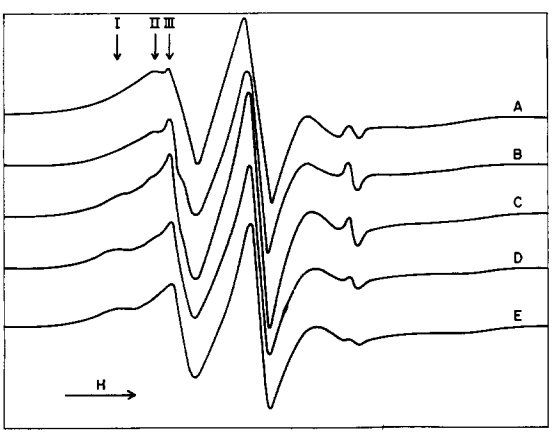

Fig. 2. EPR spectra of bovine erythrocyte ghost membrane labeled with methyl-12-doxylstearate exposed to 3-methylindole. A, 0 time; B, 5 min, 103 μg 3-methylindole/mg protein; C, 15 min, 295 μg 3-methylindole/mg protein; D, 30 min, 340 μg 3-methylindole/mg protein; E, 120 min, 436 μg 3-methylindole/mg protein. The spectrum of 60 min was similar to E, and the 3-methylindole concentration was 405 μg/mg protein.

In Fig. 2A, the narrow high field line is indicative of free fatty acids in solution. The methyl ester is not present in detectable concentration in the supernatant of the membrane suspension as determined by gas chromatography. The methylstearate spin labels were homogeneous by thin-layer chromatography. The supernatant of the membrane suspension yields an EPR spectrum which has the same line shape as

Fraction III. Incubation at 37 °C produces an increase simultaneously in the EPR signal of Fraction III and in the intensity of the thin-layer chromatography spot of 12-doxylstearate. The appearance of free fatty acid is apparently due to hydrolysis of the methyl ester. At high concentrations of 3-methylindole the free fatty acid is intercalated into the membrane.

The control EPR spectrum of methyl-16-doxylstearate intercalated in erythrocyte membranes is characteristic of weakly immobilized probes with isotropic averaging [20]. The EPR spectra of methyl-16-doxylstearate intercalated in erythrocyte membranes in the presence of 103 μg 3-methylindole per mg protein are shown in Fig. 3. In contrast to the spectral changes observed with the methyl-5-doxylstearate and methyl-12-doxylstearate, the major fraction of methyl-16-doxylstearate is not perturbed by the inclusion of 490 μg 3-methylindole per mg protein in the membrane (Fig. 3B). At the higher concentration of 3-methylindole, a strongly immobilized component is present. The strongly immobilized component is observed at high concentrations with both the methyl-12-doxylstearate and the methyl-16-doxylstearate probes. A small fraction of the methyl esters appear to hydrolyze yielding free fatty acids in solution.

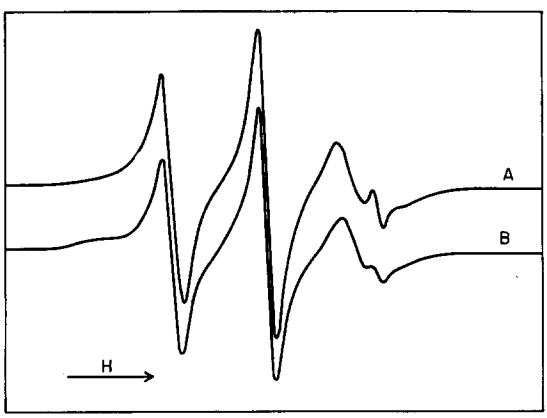

Fig. 3. EPR spectra of bovine erythrocyte membranes labeled with methyl-16-doxylstearate exposed to 3-methylindole. A, 5 min, 103 μg 3-methylindole/mg protein; B, 120 min, 460 μg 3-methylindole/mg protein.

The effects of 3-methylindole on ghost membrane protein

The EPR spectra of maleimide spin-labeled ghosts are shown in Fig. 4 and are similar to those obtained by Sandberg et al. [12]. The ratio of the height of strongly-immobilized (I) to weakly immobilized (II) low field peaks is used as an indication of the changes in population of spin labels in states I and II. The uptake of 3-methylindole by the maleimide spin-labeled ghost membranes is shown in Fig. 5. The amount of 3-methylindole in the membrane increases with the incubation time and reaches a plateau at 30 min. The strongly immobilized peaks are resolved as the 3-methylindole concentration increases in the ghost membranes. The ratio increased from 0.37 to 0.50 while the 3-methylindole concentration in the membrane increased from 0 to 390 μg/mg of membrane protein (Fig. 5). A threshold is apparent in the plot of the

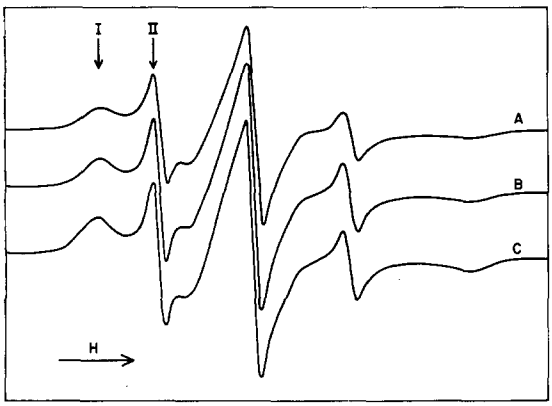

Fig. 4. EPR spectra of bovine erythrocyte membranes labeled with maleimide spin labels exposed to 3-methylindole. A, 0 time; B, 5 min, 105 μg 3-methylindole/mg protein; C, 120 min, 394 μg 3-methylindole/mg protein.

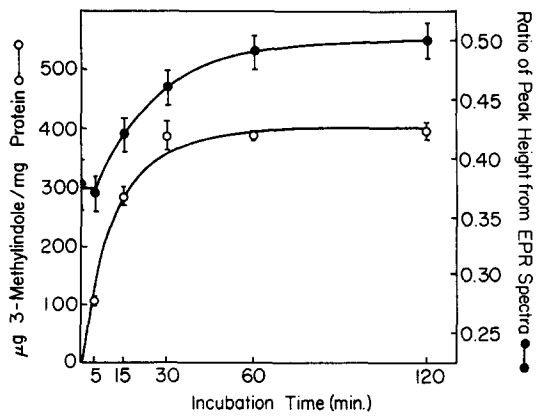

Fig. 5. The uptake of 3-methylindole by bovine erythrocyte ghost membrane with SH groups spin labeled with maleimide and the ratios of strongly immobilized to weakly immobilized low field peaks of EPR spectra as a function of 3-methylindole incubation time. The experimental uncertainties of 3-methylindole concentrations and peak ratios are represented by the vertical bars.

ratio of peak heights and indicates that more than 100 μg of 3-methylindole per mg of membrane protein was present before a change was detected in the EPR spectra. The time course for the ratio of peak heights follows the general trend of the time course for the uptake of 3-methylindole after the threshold is reached. 3-Methylindole evidently destroys the fluidity of the environment of membrane protein and increases the rigidity of the protein structure.

DISCUSSION

Several laboratories have intercalated spin-labeled stearates into membranes of erythrocytes [20, 21], influenza virus [21, 22], and *Acholeplasma laidlawaii* [23] and have observed that the degree of order and rigidity in the alkyl chains decreases

as the nitroxide moiety is moved away from the polar carboxyl head of the spin label. The aqueous solubility of stearic acid results in partitioning of the spin label between the membranes and the aqueous phase. The methyl ester has a negligible aqueous solubility which improves the resolution of the EPR spectra. The intercalation of the methyl ester into the erythrocyte membranes gives the same EPR spectra as the corresponding stearic acid in Fig. 3 of ref. 21. Thus the methyl ester group appears to be preferentially located at the polar interface of membrane lipid.

Other classes of small molecules have been observed to change the fluidity of the lipids in the membrane. Benzyl alcohol disrupts the polar head region of erythrocyte membranes [11]. The anesthetics halothane and methoxyfluorane disorder sonicated egg lecithin vesicles containing 40 molar percent cholesterol, but the concentration dependence was the same for both a 6-doxyl and a 10-doxyl probe on the β-fatty acid chain of phosphatidylcholine [28]. In contrast, cholesterol increases the order of egg lecithin bilayers over at least the first 12 carbon atoms [29].

The incorporation of 3-methylindole into the membrane also perturbs the structure of the spin-labeled proteins. The spin labels are covalently bound to sulfhydryl sites [12]. Since a threshold exists for the protein maleimide spin-labeled membranes but not for methyl-5-doxylstearate, the membrane lipids may be the site of binding of 3-methylindole which produces the detectable structural changes. EPR spectral changes indicate that the mobility of the spin label on the proteins is reduced. The experimental results do not suggest a single mechanism. One possible explanation is that the 3-methylindole interacts with the protein either at the location of spin label or 3-methylindole intercalates into the hydrophobic region of membrane integral proteins which induces a conformational change. Another explanation is that the structure of the protein is changed by a change in the fluidity of the surrounding lipid bilayer. The experimental data do not distinguish among these possibilities. It is possible that the strongly immobilized methyl-12-doxylstearate and methyl-16-doxylstearate components are bound to membrane proteins after a conformational change has occurred. In the case of cytochrome oxidase, a bound lipid layer surrounds the enzyme in the native state [11, 27].

Biological membranes play a crucial role in almost all cellular phenomena. The destruction of cells in physiological and pathological processes is often related to the alterations in the integrity of the membrane [30]. These experimental results suggest a direct physical interaction of 3-methylindole with erythrocyte membranes, resulting in structural changes.

The concentration of 3-methylindole used in these experiments was similar to those used in in vitro studies involving erythrocyte hemolysis [5] and protozoa rupture [6]. Large doses of 3-methylindole were given to cattle and goats (0.06 and 0.2 g/kg body weight) but the concentration in the lung cells is not known.

Further studies are necessary to determine whether or not 3-methylindole has a direct effect on the lung and whether pathological lesions result from changes in membrane structure induced by 3-methylindole.

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