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ERYTHROSINE B (RED DYE NO. 3) MEDIATED OXIDATION-REDUCTION IN BRAIN MEMBRANES

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SUMMARY: The results of experiments presented show that Erythrosine B (red dye No. 3) propagates oxidation-reduction events occurring outside brain membranes into the interior of the membrane bilayer. The experiments demonstrate that the nitroxide group of membrane spin-labels are reduced via an erythrosine B transient species formed by the action of peroxidase and H_2O_2. The erythrosine B transient species is permeable into the membrane bilayer and evidence indicates that it most likely is a free radical of the dye. These results may have important implications in understanding the biological action of erythrosine B.

INTRODUCTION

Erythrosine B (FD & C Red No. 3) has been implicated in hyperkinesis and learning disabilities among children. Although studies to prove this hypothesis have been inconclusive, recent studies show that large doses of a mixture of food dyes impaired learning in hyperactive children (1) and small doses elicited hyperactive behavior in a few children (1). Lafferman and Silbergeld (2) demonstrated that erythrosine B inhibited dopamine transport into rat caudate synaptosomes. Also, Logan and Swanson (3) demonstrated that a mixture of seven food dyes inhibited accumulation of eight neurotransmitters or neurotransmitter precursors by rat brain homogenate. They showed (3) that erythrosine B was the only dye which inhibited dopamine accumulation; effective concentrations were as low as 1 μg/ml. This work was recently critized by Mailman et al. (4). Nevertheless, Mailman et al. (4) did show that erythrosine B inhibited dopamine transport into crude rat striatum synaptosomes, eventhough dopamine transport was dependent upon the amount of synaptosomes present. Interestingly, also it has been shown that erythosine B attenuated the effect of punishment in a "conflict" paradigm (4). It is

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known that erythrosine B interacts with neuronal membranes (5) and neuromuscular membranes (6), increasing the resting membrane potential by increasing the potassium conductance of buccal ganglia of a marine mollusc (5). The work reported here demonstrate that erythrosine B mediates into the hydrocarbon phase of the bilayer oxidation-reduction events initiated outside the membrane. This was shown by nitroxide spin label quenching (reduction) in brain membranes. This work demonstrates that it is a transient erythrosine B intermediate, most likely a free radical of the dye that is the active component. This demonstrated capacity to interact with brain membranes in an oxidative-reductive capacity may be of importance in understanding the biological action of erythrosine B.

MATERIALS AND METHODS

Brain liposomes were prepared by brief sonication in potassium phosphate buffer (pH 7.4) of a total lipid extract from rat brain. Liposomes were spin-labeled by swirling the liposomal suspension over the inside surface of a round bottomed flask upon which the 5-nitroxide-stearate spin label had been deposited by evaporation using a vacuum pump to remove the solvent, chloroform. The ratio of phospholipid to spin-label was approximately 100 to 1 assuming that all of the label molecules were incorporated.

Synaptosomes from rat brain caudate and cerebral cortex were prepared by the Cotman and Matthews procedure (7). Spin-labeling of the synaptosomes were carried out according to the methods used for the brain liposomes.

RESULTS AND DISCUSSION

Data illustrating the general results are presented in Figure 1. Brain liposomes were spin-labeled with the membrane spin probe 5-doxylstearic acid (1-oxyl-2,2-dimethyloxazolidine derivative of 5-keto stearic acid, 5NS). When a suspension containing 5NS labelled liposomes, horse radish peroxidase (HRP) and erythrosine B (ERY-B) was exposed to hydrogen peroxide there was a rapid quenching of the nitroxide electron spin resonance spectrum. All components HRP, ERY-B, and H_2O_2 were necessary to achieve quenching of the free radical signal of the nitroxide spin label. A combination of any two of the three were ineffective. In addition, any one component by itself was without effect. HRP plus H_2O_2 only had slight if any effect on the

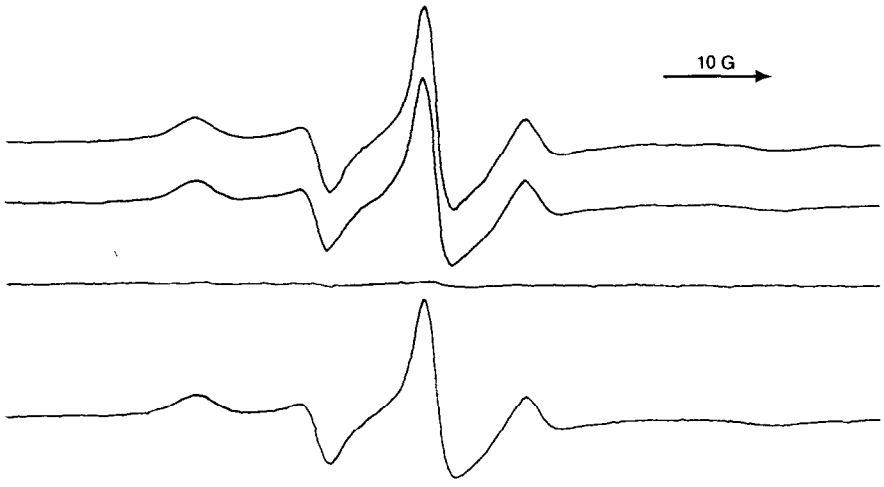

Figure 1. Erythrosine B (ERY-B) in combination with horseradish peroxidase (HRP) and H_2O_2 mediated quenching of nitroxide free radical spin-label inside the membrane bilayer. The top trace shows the electron spin resonance spectrum of the spin-labeled liposomes. The second trace down shows the spectrum obtained from a liposomal suspension that had been present when HRP (Type VI from Sigma Chemical Co., at a total amount of 0.1 mg/ml) was reacted with H_2O_2 (0.03% final amount). The third trace down shows the spectrum obtained from the liposomal suspension that had been present when HRP and ERY-B (0.87 mg/ml final concentration) had reacted with H_2O_2. In the lowest trace, the spectrum was taken of the liposomal suspension after the HRP, ERY-B and H_2O_2 had been reacted in a separate tube and then placed in contact with spin-labeled liposomes 5 min after initiating the peroxidase reaction. The instrument settings were similar to those described in Figure 2 only the gain was an order of 10 lower.

nitroxide signal size. If in contrast to the protocol of the experiments described above, a separate solution containing HRP, ERY-B and H_2O_2 was allowed to react for 5 min and then this solution placed in contact with the 5NS liposomes, there was only a slight if any reduction of the liposomal nitroxide free radical signal. These results illustrate that quenching of the nitroxide free radical in the lipid bilayer depends on a transient species of ERY-B produced by the action of peroxidase and H_2O_2.

Equivalent and consistent results have been obtained with all natural and synthetic membranes tested thus far including rat caudate and cerebral cortex synaptosomes. Separate experiments have demonstrated that the double

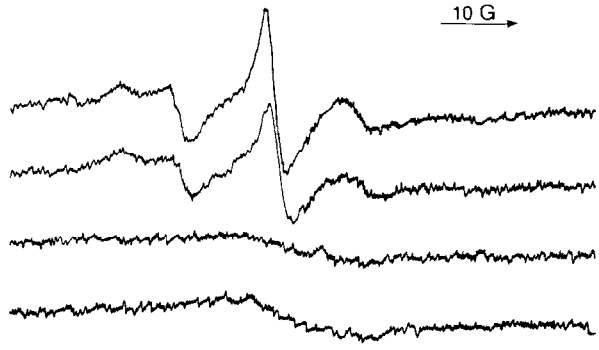

Figure 2. Experiment demonstrating that double bonds in the bilayer are not necessary for achieving the ERY-B mediated nitroxide spin-label quenching and that quenching was achieved with nitroxide spin-labels exclusively bilayer localized. Ditetradecanoyl-L-α-lecithin, obtained from Supelco Co. and 5-nitroxide-stearate spin labeled on the β-chain of phosphatidyl choline, obtained as a gift from Dr. Ian C.P. Smith, were plated at a 100/1 molecular ratio on a glass surface by vacuum pump evaporation of the chloroform solvent. Liposomes were formed by sonication (30 min total time by intermediate bursts) in a 0.05 M pH 7.5 potassium phosphate buffer. The top electron spin resonance spectrum shows the spin-labeled liposomes only. The second spectrum down shows the spin-labeled liposomes which had been present during a reaction of horse radish peroxidase (0.0125/ml) plus H_2O_2 (0.0014%). The third trace down shows the spectrum of the solution where HRP and H_2O_2 and ERY-B (1.0 mg/ml) had been reacted with no liposomes present. The fourth trace down shows the spectrum of the solution where HRP, H_2O_2, ERY-B had reacted in the presence of spin-labeled liposomes. The temperature of the solutions during the spectra recording was 37°. Scan time 30 min/100 gauss, gain 2.5×10^4, 25 mWatt, 2 gauss modulation amplitude at 100 KHz, response time 3 sec. The arrow indicates the direction of low to high magnetic field and the length indicates 10 gauss.

bonds present in fatty acid esters of natural membranes are not a prequisite to achieve the ERY-B mediated peroxidase-H_2O_2 quenching of membrane localized nitroxide spin labels. These results are shown in Fig. 2. Thus we have shown using spin-labeled phosphatidyl choline (5-nitroxyl stearate on the β-chain) incorporated into liposomes formed from tetradecanoyl-phosphatidyl choline that equivalent results were obtained as those presented in Figure 1. Since the nitroxide of spin-labeled phosphatidyl choline resides exclusively in the membrane bilayer this eliminates the possibility that the results with 5-nitroxide stearate spin label could be explained by the spin label flipping in and out of the bilayer phase.

In addition to quenching of nitroxide groups positioned within the membrane bilayer, we have demonstrated that the nitroxide free radical of a freely soluble compound is quenched by the HRP/H_2O_2 generated transient ERY-B species. This was demonstrated by utilizing 30% methanol to solubilize the 5-doxyl-stearic-acid spin label. I found that the combination of HRP plus H_2O_2 caused a loss of about 50% of the freely tumbling nitroxide whereas if ERY-B was present in combination with HRP and H_2O_2 then a complete loss of nitroxide signal was observed. The combination of HRP plus ERY-B in the absence of H_2O_2 actually caused a slight enhancement of the nitroxide signal.

Experiments conducted indicate that the ERY B mediated HRP/H_2O_2 nitroxide quenching is due to a chemical reduction of the nitroxide free radical. This was demonstrated by utilizing the water soluble nitroxide, tempol (4-hydroxyl-2,2,6,6-tetramethylpiperidine-1-oxyl) in the presence of limiting amounts of H_2O_2, in the HRP/H_2O_2/ERY-B system such that only approximately 2/3 of the nitroxide was quenched, I found that bubbling with oxygen resulted in a progressive reappearance of the original full nitroxide signal within 50 min after the HRP/H_2O_2/ERY-B reaction. This indicates that the nitroxide is reduced by the ERY-B transient species and is then reoxidized by oxygen in accordance with the known response of reduced nitroxide compounds to oxygen. It is known that nitroxide spin labels are reduced, and hence become electron spin resonance invisible, in natural membranes containing reduced pyridine nucleotide dehydrogenase enzymes (8, 9).

The transient ERY-B species responsible for reduction of the nitroxide group within the membrane bilayer is most likely a free radical of ERY-B. This conclusion is based upon the proven normal three step cycling of HRP (10) in producing free radicals of many electron donor compounds such as ascorbate (11), reductate (11), the carcinogen N-hydroxy-2-acetylaminofluorene (12, 13) and many phenolic compounds, to mention a few. Experiments that I have conducted to date designed to observe the ERY-B free

radical have shown that a free radical is formed, but it decays rapidly and therefore has not yet been characterized. Other experiments where competing electron donors such as ascorbate were added to the HRP/H_2O_2/ERY-B system demonstrated that these substrates prevented membrane localized nitroxide spin label reduction. Addition of the enzyme superoxide dismutase had no effect on the ERY-B mediated HRP plus H_2O_2 reduction of the nitroxide spin-labels. Addition of KO_2 to spin labeled membranes in the presence or absence of ERY-B had no effect on the nitroxide spin-label. Also, utilizing the 5-doxyl-stearic acid spin-label, I have found that the microviscosity of rat brain synaptosomal membranes is not changed by ERY-B addition.

The results presented in this report demonstrate for the first time that the commonly used red dye, ERY-B, mediates into the membrane bilayer oxidation-reduction events occurring outside the membrane. The consequences of this are not known at the present time; however it does appear possible that there may be biological consequences since several oxidative-reductive processes, such as mitochondrial electron flow and steroid and drug metabolism in endoplasmic reticulum, occur in natural membranes and hence it is possible that ERY-B may either interfere or perhaps be converted to free radicals during these processes. The effect of ERY-B on these processes and other brain membrane mediated functions needs further study.

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REFERENCES

1. Swanson, J.M. and Kinsbourne, M., (1980) Science 207: 1485-1487.
2. Lafferman, J.A. and Silbergeld, E.K. (1979) Science 205: 410-412.
3. Logan, W.J. and Swanson, J.M. (1979) Science 206: 363-364.
4. Mailman, R.B., Ferris, R.M., Tang, F.L.M., Vogel, R.A., Kilts, C.D., Lipton, M.A., Smith, D.A., Mueller, R.A. and Breese, G.R. (1980) Science 207: 535-537.
5. Levitan, H. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 2914-2918.

6. Augustine, G.H., Jr. and Levitan, H. (1980) Science 207: 1489-1490.
7. Cotman, C.W. and Matthews, D.A. (1971) Biochim. Biophys. Acta 249: 380-394.
8. Bladassare, J.J., Robertson, D.E., McAfee, A.G. and Ho, C. (1974) Biochem. 13: 5210-5214.
9. Hampton, M.J., Floyd, R.A., Clark, J.B. and Lancaster, J.H. (1980) Mutation Res. 69: 231-239.
10. Chance, B. (1952) Arch. Biochem. Biophys. 41: 416-424.
11. Yamazaki, I., Mason, H.S. and Piette, L. (1960) J. Biol. Chem. 235: 2444-2449.
12. Bartsch, H. and Hecker, E. (1971) Biochim. Biophys. Acta 237: 567-578.
13. Floyd, R.A., Soong, L.M. and Culver, P.L. (1976) Cancer Res. 36: 1510-1519.