

Halothane and Isoflurane Alter Phospholipid Transmethylation in Rat Brain Synaptosomes

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The mechanism of action of inhalational anesthetics is unknown, but neuronal membrane alteration is a favored hypothesis. Since phospholipid methylation and translocation play a key role in the transmission of biologic signals across cell membranes, we examined the effect of two commonly used halogenated anesthetics, halothane and isoflurane, on phospholipid methylation in rat brain synaptosomes. Using S-adenosyl-L-[³H-methyl]methionine as a donor, we found a two-fold increase in ³H-methyl incorporation into phospholipids in synaptosomes taken from rats exposed to concentrations that just abolish pain response, but not in rats exposed to higher or lower concentrations. Methylation was not increased in rats newly recovered from anesthesia. Halothane added to synaptosomes taken from rats not previously exposed to anesthetics stimulated ³H-methyl incorporation over a wide range of concentrations. Enhancement of phospholipid methylation by halothane and isoflurane may effect an alteration of neural signal transduction that results in the anesthetic state. (Key words: Anesthetics, volatile: halothane, isoflurane. Brain: synaptosomes, phospholipid transmethylation. Phospholipids: phosphatidylethanolamine, phosphatidylcholine.)

THE INTRODUCTION of inhalational anesthetics a century and a half ago marks a major medical and pharmacologic achievement, but how these agents work remains unknown. In recent years neuronal membranes have been favored as a possible site of anesthetic effect, either by nonspecific, biophysical interaction of anesthetics with membrane lipids¹⁻⁴ or by direct anesthetic induction of conformational changes in membrane proteins.⁵⁻⁶

Neuronal membranes, like all cell membranes, consist of a phospholipid bilayer in which biochemically active proteins are embedded.⁷ Hirata and Axelrod⁸ have provided important information on the interaction between biologic signal transmission and membrane phospholipid transformation. There is now strong evidence that enzymatic methylation of membrane phospholipids facilitates the transduction of receptor-mediated signals through cell membranes.⁸ Phospholipid methylation converts phosphatidylethanolamine (PE) to phosphatidylcho-

line (PC) by successive N-terminal methylations, with S-adenosyl-L-methionine (SAM) as a methyl donor.⁹ Two membrane-bound enzymes involved in this reaction have been found in a variety of cells¹⁰⁻¹⁴ and in brain synaptosomes.¹⁵ The first enzyme, methyltransferase I, converts PE to phosphatidyl-N-monomethylethanolamine (PME); has a high affinity for SAM ($K_m = 1-2 \mu M$); and controls the rate-limiting step of the methylation reaction. Methyltransferase II catalyzes the successive methylation of PME to phosphatidyl-N,N-dimethylethanolamine (PDE) and to PC.

A critical link between membrane phospholipid methylation and membrane receptor stimulation has been established by Hirata *et al.*¹⁶ in studies with rat reticulocytes. Activation of beta-adrenergic receptors on the surfaces of these cells by catecholamines results in stimulation of [methyl-³H]SAM incorporation into phospholipid, with the associated translocation of the methylated product from the inner to the outer membrane surface. The reverse also occurs: stimulation of methylation by addition of S-adenosyl-L-methionine increases the number of beta-adrenergic binding sites.

We examined the effect of two commonly used halogenated inhalational anesthetics, halothane and isoflurane, on phospholipid methylation in rat brain synaptosomes. We report here that induction of the anesthetic state with these agents increases synaptosomal phospholipid methylation.

Materials and Methods

ANESTHETIC PROCEDURE

Animal use was approved by the Animal Care Committee of Vanderbilt University. Male Sprague-Dawley rats weighing 285-457 g were anesthetized with halothane or isoflurane in warmed, humidified air and oxygen ($FI_{O_2} = 0.3$). Vaporizers were calibrated by gas chromatographic measurements of anesthetic concentrations, and additional measurements of concentration were made during anesthetic administration. Induction of anesthesia was carried out in rats breathing 2.4% halothane or 2.9% isoflurane in a 3-l chamber. When immobilized, each rat was removed from the chamber, and the clock was started. Anesthesia was delivered *via* a T-piece circuit to a Tygon cylinder (depth 2.5, height 5 cm) stoppered at one end. The gases entered through a lateral opening in the cyl-

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inder and exited through an opening at the stoppered (distal) end. With the rat prone, the cylinder fit snugly over the muzzle, and when held in place with both hands, thumbs thrusting the mandible down and forward, it delivered, under careful observation, an adequate tidal volume.¹⁷

Intermittent positive pressure in the system was developed (55–60 breaths per min) by occluding gas outflow with a solenoid valve. The level of anesthesia was assessed by the response to intermittent application of a tail clamp. Anesthetic concentration was adjusted so that the dose delivered was just above the level at which a tail flick could be induced by clamping, thus establishing a minimum effective dose (MED) for each rat. The MED, mean \pm standard error of the mean (SEM), for halothane was $1.43 \pm 0.08\%$ ($n = 8$) and for isoflurane was $1.90 \pm 0.07\%$ ($n = 8$). After 20 min, the animal was decapitated and the brain immediately placed in 0.32 M sucrose at 4° C. A second group of rats was similarly anesthetized (two with halothane and two with isoflurane) and then allowed to recover before decapitation. Recovery was considered to have occurred when a rat, after briefly moving around his cage, began grooming. This activity began 20–23 min after the cessation of anesthetic administration. Control animals ($n = 8$) were decapitated after a 20-min exposure to a warmed, humidified air–oxygen mixture ($F_{I_{O_2}} = 0.3$).

Studies at four additional concentrations were carried out with halothane. Rats ($n = 4$) were exposed to 0.50% halothane in a 3-l chamber for 20 min and remained somnolent but moved when stimulated. A second group was anesthetized with halothane as described above, and the concentration was adjusted so that the animal remained immobile but clamping resulted in movement of the tail. The mean halothane concentration for this group was $0.92 \pm 0.03\%$ ($n = 3$). A third group was anesthetized with halothane, and an MED was established for each rat, as described above. After 10 min, the halothane concentration increased 0.5%, and anesthesia was continued for another 10 min. The mean concentration for this group was $1.92 \pm 0.08\%$ ($n = 4$). A fourth group ($n = 4$) was anesthetized and maintained under anesthesia with 2.45% halothane. These animals became slightly cyanotic after 13–15 min, presumably because of cardiac depression. Anesthetic exposure was terminated with the appearance of cyanosis. Rats in all groups were decapitated immediately after anesthetic exposure was discontinued.

BRAIN SYNAPTOSOME PREPARATION

Synaptosomes were isolated from brain homogenates according to the method described by Cotman.¹⁸ Excised brains were placed in 0.32 M sucrose (Ultrapure, ICN, Cleveland, OH) at 4° C. They were blotted, weighed,

minced, and then homogenized (Ultra Turrax, Tekmar, Cincinnati, OH) in cold 0.32 M sucrose (total volume 10 ml/g blotted brain). The homogenate was centrifuged at 1000 g for 5 min in a Beckman model J2-21 refrigerated preparative centrifuge with a JA-17 rotor. The supernatant fraction was centrifuged at 15,000 g for 15 min, and the resultant pellet was resuspended in 8 ml 0.32 M sucrose. Four-milliliter aliquots of this suspension were applied to gradients consisting of 6-ml layers each of 4%, 6%, and 13% Ficoll (400 kD, Sigma, St. Louis, MO) in 0.32 M sucrose. Gradients were centrifuged at 63,580 g for 45 min in a 55.2 Ti rotor in a Beckman L8-55M ultracentrifuge. The synaptosomal fraction was collected from the 6–13% interface, diluted with 10 ml 0.32 M sucrose, and centrifuged again at 63,580 g for 20 min. The resultant pellet was suspended in 0.32 M sucrose.

SYNAPTOSOMAL PHOSPHOLIPID METHYLATION

Synaptosomal phospholipid methylation was measured by the incorporation of tritiated methyl groups from S-adenosyl-L-[³H-methyl]methionine (Amersham, Arlington Heights, IL) into PME, PDE, and PC, according to the procedures described by Crews *et al.*¹⁵ and Sastry *et al.*¹⁴ The incubation mixture consisted of 0.2 mg synaptosomal protein, 50 mM Tris-glycylglycine buffer (*pH* 8.0), 5 mM MgCl₂, 0.1 mM EDTA, and 2 μ M [methyl-³H]SAM (added last to start the reaction) in a final volume of 0.1 ml. After a 30-min incubation at 37° C in a shaking water bath, the reaction was stopped by the addition of 3 ml chloroform:methanol:HCl (2:1:0.02) and shaken. The chloroform layer was washed twice with 2 ml 0.1 M KCl in 50% methanol, and after the addition of 100 mg anhydrous Na₂SO₄, was stored at –20° C. Phospholipids in concentrated aliquots of the chloroform extract were separated by thin layer chromatography (TLC) on silica gel G plates (Fisher, Norcross, GA) developed in chloroform:propionic acid:n-propanol:water (2:2:3:1). PME, PDE, and PC fractions were identified by comparison with standards, scraped into scintillation vials containing Cyto Scint (ICN Radiochemicals, Irvine, CA), and counted in a Beckman LS 3801 Beta Counter. No significant radioactivity was found to co-chromatograph with lysophosphatidylcholine (LPC) or lysophosphatidylethanolamine (LPE). The conditions of the assay were optimum for methyltransferase I, the first and rate-limiting enzyme in the phospholipid methylation pathway. Therefore, results were expressed as total product of methyltransferase I formed, *i.e.*, as femtomoles of ³H-methyl groups incorporated into PE to form ³H-PME. Part of the ³H-PME was further methylated to form ³H-PDE and ³H-PC. Femtomoles of ³H-PDE and ³H-PC were divided by 2 and 3, respectively, to obtain femtomoles of ³H-PME which

had been further methylated. These corrected values were added to measured femtomoles of ^3H -PME formed to calculate total methyl group incorporation into synaptosomal PE.

IN VITRO HALOTHANE EXPOSURE

To examine the effect of an inhalational anesthetic on phospholipid methylation in isolated subcellular fractions, synaptosomes were prepared from brain homogenates from unanesthetized rats and incubated with one of six concentrations of halothane—0.25, 0.50, 0.75, 1.0, 2.0, or 3.0 mM. Initially, halothane was dissolved in 50 mM tris-glycylglycine buffer, pH 8.0, 5 mM MgCl_2 , and 0.1 mM EDTA, at concentrations of 0.5, 1.0, 1.5, 2.0, 4.0, and 6.0 mM and then diluted 1:1 in reaction medium containing 2 μM S-adenosyl-L- ^3H -methyl]methionine, 0.2 mg synaptosomal protein, and buffer. The reaction volume of 0.2 ml was incubated in a 1.5-ml glass vial with a Teflon[®]-lined screw cap. Samples were processed as described above.

In a second set of experiments, synaptosomes prepared from unanesthetized rats were incubated as described in the section above, except that the incubation mixture was placed in open-necked, uncapped glass vials (diameter = 5 mm, height = 10 mm). Halothane was not added to the mixture directly. Instead, the vials were placed in a Dubnoff shaker under a gassing hood and shaken gently at 37° C. Halothane, in a warmed, humidified air and oxygen mixture ($\text{FI}_{\text{O}_2} = 0.3$), was delivered under the hood in concentrations of 0.5, 1.4, 1.9, or 2.4% for 30 min. Delivered concentrations were confirmed by gas chromatography. The methylated phospholipids were extracted and processed as described above.

STATISTICAL ANALYSIS

Data were compared by analysis of variance and the Tukey multiple comparison procedure.¹⁹ Statistical significance was inferred if $P < 0.05$.

Results

As shown in figure 1, total ^3H -methyl group incorporation, (femtomoles per milligram protein per 30 min) into synaptosomal phospholipids from rats exposed to one MED of halothane or isoflurane was increased two-fold, to 978 ± 48 and 921 ± 86 fmol, respectively, compared with control rats at 402 ± 38 fmol, $P < 0.01$. Recovery from anesthesia was associated with values (267 ± 15 fmol) not significantly different from control. Addition of 100 μM of S-adenosyl-L-homocysteine (SAH), an inhibitor of phospholipid methylation,²⁰ reduced methyl group in-

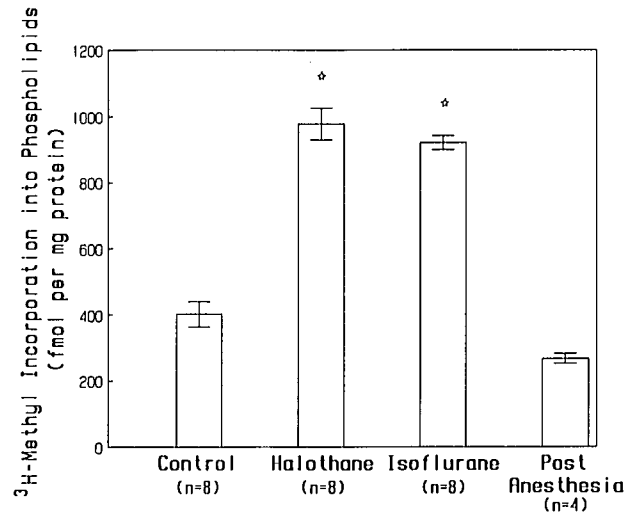


FIG. 1. Anesthetic effect on synaptosomal phospholipid methylation. Bars indicate mean values with SEM; n = number of rats. Anesthetic concentration was adjusted, as described in the text, so that a minimum effective dose (MED) was delivered, i.e., just above the level at which a tail flick could be induced by clamping. This concentration averaged $1.43 \pm 0.08\%$ for halothane and $1.90 \pm 0.07\%$ for isoflurane. *Differs from control and postanesthesia rats, $P < 0.01$.

corporation in all synaptosomal preparations to concentrations below initial control values.

The component products of synaptosomal transmethylation—PME, PDE, and PC—all showed a similar pattern of increased ^3H -methyl incorporation with anesthetic exposure (table 1). Since incubation conditions in these studies were set to facilitate methyltransferase I,⁸ the predominant product in all treatment groups was PME. Proportions of component methylated products in each of the four treatment groups did not differ significantly from control.

Halothane and isoflurane increased methyl incorporation by two-fold into myelin phospholipids as well, 809 ± 48 and 801 ± 53 fmol, respectively, versus control rats at 403 ± 17 fmol, $p < 0.01$. Microsomes from the brains of rats exposed to one MED of halothane or isoflurane incorporated ^3H -methyl groups into phospholipids at a rate 50% higher than did control microsomes after isoflurane (1416 ± 82 vs. 925 ± 60 fmol, $P < 0.05$) and at a rate not significantly changed after halothane (1149 ± 25 fmol).

Figure 2 compares phospholipid methylation in brain synaptosomes from rats exposed to 1 MED of halothane with results from animals exposed to concentrations above and below that level. It is clear that the dosage range in which increased phospholipid methylation occurs is quite narrow, since only in the very middle range, at precisely 1 MED, was it manifest.

TABLE 1. Anesthetic Effect on Incorporation of ³H-methyl Groups into Component Products of Synaptosomal Transmethylation

Phospholipid	Treatment Group			
	Control	Halothane	Isoflurane	Postanesthesia
PME	217 ± 26	517 ± 41*	428 ± 52*	152 ± 9
PDE	131 ± 11	311 ± 16*	339 ± 28*	73 ± 7
PC	54 ± 2	150 ± 10*	154 ± 14*	42 ± 3

Concentrations are femtomoles per milligram protein.

* Differ significantly from control and postanesthesia, *P* < 0.01. Methods are described in the text.

To examine the effect of an inhalational anesthetic on phospholipid methylation in isolated subcellular fractions, synaptosomes were obtained from unanesthetized rats and incubated *in vitro* with one of six concentrations of halothane—0.25, 0.50, 0.75, 1.0, 2.0, or 3.0 mM. The resulting dose-response curve for incorporation of ³H-methyl groups into total phospholipids is shown in figure 3. Phospholipid methylation increased with increasing halothane concentration, but leveled off at approximately 1 mM with a ³H-methyl incorporation rate in the same range as that observed in synaptosomes from anesthetized animals.

Exposure of synaptosomes from unanesthetized rats to one of five partial pressures of halothane produced a somewhat similar dose-response curve (fig. 4). Methyl group incorporation was not increased above control values at 0.50%, but was maximally increased at 1.00 and 1.40% and was moderately increased at 1.90 and 2.40% halothane. The very narrow range of stimulation obtained

with *in vivo* exposure (fig. 2) was not seen, but incorporation did decrease at higher halothane concentrations with both types of *in vitro* exposures.

Discussion

This study demonstrates that a molecular change in brain plasma membranes occurs with induction of a pain-free state, *i.e.*, anesthesia, and is no longer evident after recovery from that state. *In vitro* exposure of brain synaptosomes to varying doses of halothane produced a similar membrane response. Since methyltransferase I is the rate-limiting enzyme of phospholipid methylation and since the response we observed occurred under incubation conditions favoring methyltransferase I, this enzyme is a likely candidate for an anesthetic site of action. Perhaps halothane and isoflurane induce conformational changes that enhance methyltransferase activity. Alternatively, these agents may activate as yet unidentified membrane

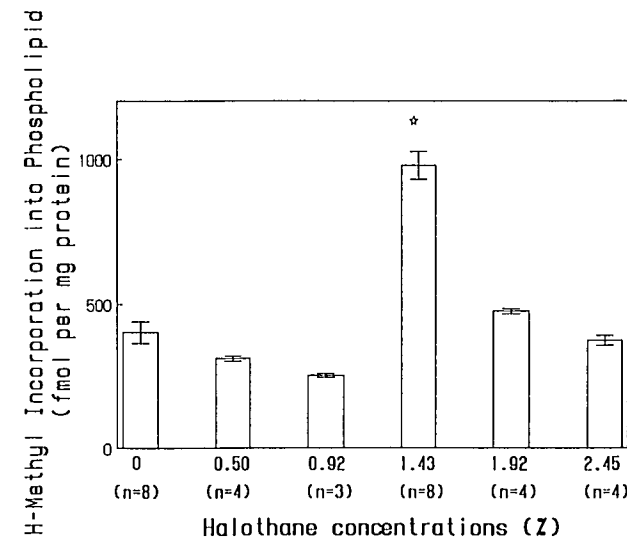


FIG. 2. Effect of different halothane concentrations on synaptosomal phospholipid methylation. Anesthetic concentration was adjusted as described in the text; 1.43 ± 0.08% represents a minimum effective dose (MED). *Differs from control value, *P* < 0.01.

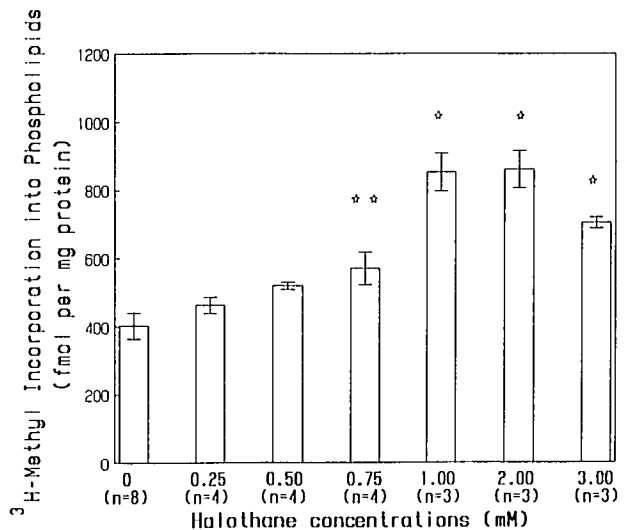


FIG. 3. Effect of *in vitro* halothane on synaptosomal phospholipid methylation. Halothane was added to the synaptosomal incubation media as indicated; n = number of rats from which synaptosomes were isolated without prior exposure to anesthetic. *Differs from control value, *P* < 0.01; **Differs from control value, *P* < 0.05.

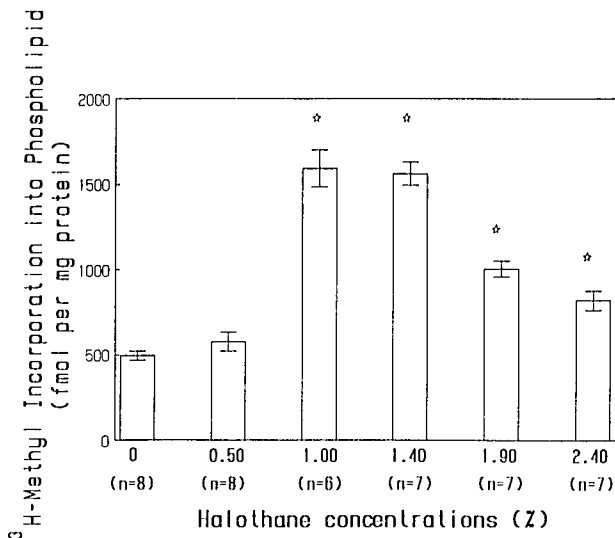


FIG. 4. Effect of *in vitro* halothane on synaptosomal phospholipid methylation. Synaptosomal incubation mixtures were exposed to different concentrations of halothane vapor as indicated; n = number of rats from which synaptosomes were isolated without prior exposure to anesthetic. *Differs from control value, $P < 0.01$.

receptors with secondary stimulation of methyltransferase.¹⁶

The narrow *in vivo* range of halothane concentration associated with increased phospholipid methylation (fig. 2) suggests that anesthetic interaction with a single enzyme represents an oversimplified hypothesis. Activation of methyltransferase I may be one of a series of membrane molecular responses to anesthetics. Some of these responses may occur at different anesthetic concentrations. Halogenated anesthetics can rapidly inhibit protein synthesis.^{17,21} It therefore is possible that high *in vivo* halothane concentrations preferentially inhibit synthesis of SAH hydrolase, which may turn over rapidly, allowing accumulation of SAH, an inhibitor of phospholipid methylation. Inhibition of enzyme synthesis could not, of course, account for diminished methylation when isolated synaptosomes are exposed to high halothane concentrations *in vitro*, since protein synthesis does not occur in this subcellular fraction. Alternatively, high concentrations of halothane may directly inhibit SAH hydrolase.

Other mechanisms possibly accounting for the lack of measured effect of high concentrations of halothane include maximal enzyme (or putative receptor) stimulation with resultant depletion of precursor PE or prior accumulation of unlabeled methylated phospholipids in synaptosomal membranes. A synaptosomal membrane in which maximal phospholipid transmethylation and translocation has taken place might remain in a prolonged "transduced state" despite its inability to incorporate fur-

ther quantities of [methyl-³H]SAM *in vitro*. Linkage of this transduced state with membrane ion transport⁸ or with neurotransmitter release²² may represent the effector pathway of anesthetic action.

Halothane and isoflurane alter somatosensory, auditory, and visual evoked potentials, indicating that these anesthetic agents may interfere with axonal impulse transmission. Both synaptosomal and myelin membranes have phospholipid composition similar to that of other plasma membranes. The occurrence of phospholipid methyltransferase in myelin and the influence (primary or secondary) of anesthetics on its activity suggest that phospholipid methylation also may affect axonal impulse conduction. On the other hand, halothane did not significantly alter phospholipid methylation in brain microsomes. This finding is not necessarily inconsistent with results obtained with synaptosomes and myelin, since microsomal membranes may not be directly involved in intercellular communication.

Whatever complexities emerge regarding halothane and isoflurane interaction with membrane elements, our current results suggest that enhancement of phospholipid methylation is a clearly defined step in the alteration of biologic signal transmission that results in the anesthetic state. Precisely how transmission is modified remains to be established.

References

1. Miller KW, Pang, KW: General anaesthetics can selectively perturb lipid bilayer membranes. *Nature (London)* 263:253-255, 1976
2. Trudell JR: A unitary theory of anesthesia based on lateral phase separations in nerve membrane. *ANESTHESIOLOGY* 46:5-10, 1977
3. Jannoff AS, Pringle MJ, Miller KW: Correlation of general anesthetic potency with solubility in membranes. *Biochim Biophys Acta* 649:125-128, 1981
4. Halsey MJ, Wardley-Smith B, Green CJ: Pressure reversal of general anesthesia: A multi-site expansion hypothesis. *Br J Anaesth* 50:1091-1097, 1978
5. Richards CD, Martin K, Gregory S, Keightly CA, Hesketh TR, Smith GA, Warren CB, Metcalfe JC: Degenerate perturbations of protein structure as the mechanism of anaesthetic action. *Nature (London)* 276:775-779, 1978
6. Franks NP, Lieb WR: Molecular mechanisms of general anesthesia. *Nature (London)* 300:487-493, 1982
7. Singer SJ, Nicolson GL: The fluid mosaic model of the structure of cell membranes. *Science* 175:720-731, 1972
8. Hirata F, Axelrod J: Phospholipid methylation and biological signal transmission. *Science* 209:1082-1090, 1980
9. Bremner K and Greenber DM: Methyltransferring enzyme system of microsomes in the biosynthesis of lecithin (phosphatidylcholine). *Biochim Biophys Acta* 46:205-216, 1961
10. Hirata F, Viveros OH, Dilberto EM, Jr, Axelrod J: Identification and properties of two methyltransferases in the conversion of phosphatidylethanolamine to phosphatidylcholine. *Proc Natl Acad Sci USA* 75:1718-1721, 1978
11. Hirata F, Axelrod J: Enzymatic synthesis and rapid translocation

- of phosphatidylcholine by two methyltransferases in erythrocyte membranes, *Proc Natl Acad Sci USA* 75:2348-2352, 1978
12. Hirata F, Toyoshima S, Axelrod J, Wasdal, MJ: Phospholipid methylation: A biochemical signal modulating lymphocyte mitogenesis. *Proc Natl Acad Sci USA* 77:862-865, 1980
 13. Sastry BVR, Statham CN, Axelrod J, Hirata F: Evidence for two methyltransferases involved in the conversion of phosphatidylethanolamine to phosphatidylcholine in the rat liver. *Arch Biochem Biophys* 23:211-222, 1981
 14. Sastry BVR, Statham CN, Meeks RE, Axelrod J: Changes in phospholipid methyltransferases and membrane fluidity during induction of rat liver microsomal cytochrome P-450 by phenobarbital and 3-methylcholanthrene. *Pharmacology* 23:211-222, 1981
 15. Crews FT, Hirata F, Axelrod J: Identification and properties of methyltransferases that synthesize phosphatidylcholine in rat brain synaptosomes. *J Neurochem* 34:1491-1498, 1980
 16. Hirata F, Strittmatter WJ, Axelrod J: β -adrenergic receptor agonists increase phospholipid methylation, membrane fluidity, and β -adrenergic receptor-adenylate cyclase. *Proc Natl Acad Sci USA* 76:368-372, 1979
 17. Franks JJ, Kruskal JB, Kirsch RE, Beechey APG, Morell DF, Harrison GG: Halothane decreases albumin and transferrin synthesis: Studies in the isolated, perfused rat liver, and in the intact rat. *ANESTHESIOLOGY* 68:529-533, 1988
 18. Cotman CW: Isolation of synaptosomal and synaptic plasma membrane fractions, *Methods in Enzymology*, Volume 31. Edited by Fleischer S, Packer L. New York and London, Academic Press, 1974, pp 445-452
 19. Dowdy S, Wearden S: *Statistics for Research*. New York, John Wiley and Sons, 1983, pp 251-271
 20. Mann SH, Mudd JD: Alkaloids and plant metabolism: IV. The tyramine methyltransferase of barley roots. *J Biol Chem* 238:181-185, 1963
 21. Franks JJ, Kruskal JB, Holaday DA: Immediate depression of fibrinogen, albumin, and transferrin synthesis by halothane, isoflurane, sevoflurane and enflurane (abstract). *ANESTHESIOLOGY* 71:A238, 1989
 22. Rabe CS, Williams TP, McGee, Jr R: Enhancement of depolarization-dependent release of norepinephrine by an inhibitor of S-adenosylmethionine-dependent transmethylation. *Life Sci* 27:1753-1759, 1980